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SYMPOSIUM

ON

PHYSIOLOGICAL EFFECTS OF RADIATION AT THE CELLULAR LEVEL

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1952

FOREWORD

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A large part of the cost of most scientific journals is borne by the subscribers. The publication of lengthy manuscripts in the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY would require a further increase in the subscription price or, alternatively, would reduce the number of pages which could be assigned to other authors who desire to publish articles relating to the many fields of physiology encompassed by this Journal. Accordingly, in the interest of our subscribers, it is necessary to limit the number of pages made available to any author and for the presentation of material dealing with any one subject.

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DETLEV W. BRONK

SYMPOSIUM ON
PHYSIOLOGICAL EFFECTS OF RADIATION AT THE CELLULAR LEVEL

GIVEN AT

RESEARCH CONFERENCE FOR BIOLOGY AND MEDICINE OF THE ATOMIC ENERGY COMMISSION

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CONTENTS

Introduction — ALEXANDER HOLLAENDER 9

L. R. BLINKS. Effects of radiation in marine algae 11

E. J. BOELL. The effects of radiations on respiratory metabolism. Three figures 19

ALEXANDER L. DOUNCE. The interpretation of chemical analyses and enzyme determinations on isolated cell components 43

DAVID E. GREEN. Organized enzyme systems 75

MORTON MCCUTCHEON. Problems and effects of radiation on capillary permeability. With discussion by Jacob Furth — Endothelial fragility by ionizing radiation. Three figures 113

DAVID NACHMANSOHN. Nerve function and irradiation effects. Eleven figures 137

A. K. PARPART AND J. W. GREEN. Some problems in connection with ion permeability of erythrocytes 179

C. W. SHEPPARD AND MARIAN STEWART. The direct effects of radiation on erythrocytes. Fourteen figures 189

LUDWIG VON SALLMANN. The effects of radiation on the cytology of the eye. Four figures 217

INTRODUCTION

The Fourth Annual Biology Research Conference, sponsored by the Biology Division of the Oak Ridge National Laboratory in cooperation with the Atomic Energy Commission and the Oak Ridge Institute of Nuclear Studies, dealt with the physiological effects of radiation at the cellular level. The program was introduced by Dr. Shields Warren, Director of Division of Biology and Medicine of the Atomic Energy Commission.

As at previous conferences, free and open discussion of the subject was encouraged; and an appreciable amount of the discussion is reproduced. The conference, as well as the publication of the proceedings, follows the same pattern established at the previous four conferences. All four of the symposia are published as supplements to this Journal.

1. Radiation Genetics, 1948 Conference, Vol. 35, Suppl. 1, June, 1950.
2. Radiation Microbiology and Biochemistry, 1949 Conference, Vol. 39, Suppl. 1, March, 1952.
3. Current Problems in Nucleic Acid Chemistry, 1950 Conference, Vol. 38, Suppl. 1, 1951.
4. Physiological Effects of Radiation at the Cellular Level, 1951 Conference, this suppl.

In this symposium an extensive and important discussion by Dr. Jacob Furth, of this Laboratory, is included in the open discussion on Dr. McCutcheon's article. The technical editing, as in previous proceedings, was done by Miss Eileen Slaughter.

April 1952 ALEXANDER HOLLAENDER

EFFECTS OF RADIATION ON MARINE ALGAE

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This paper reports work done on certain physiological effects of the atom bombs at Bikini, one year after the explosions.[2] The principle was to study marine algae taken from normal habitats, and from the most intensely radioactive regions which could be found on the atoll. The actual intensity one year after the explosion, was of course, rather low — two or three times background count in the milder regions up to 50 times background on the "hotter" coral heads nearest the blast site. The first, main "tidal wave" from the explosion washed directly over the reefs most used for collecting. Whether radioactive elements were deposited at that time, or later, as the silt from the lagoon was washed up, cannot be said with any certainty, since rapid evacuation followed the explosion and tests were apparently not made for some 9 months after. It may be assumed, however, that the algae were exposed to continual and, at first, fairly intense mixed radiation, which fell exponentially depending on the half life of the fission products. It is believed, from the size and appearance of the algae, that no great amount of killing occurred, since the radioactive regions appeared scarcely different from those at a distance. It does not seem possible that new growth had recolonized killed areas so quickly. For example, when the northwestern point of Bikini was first investigated by us, we were impressed by the complete absence of algae on and around the wreckage of a small boat at that point. Neither the planks, rocks upon the ribs, nor rocks within 10 feet had algal growth of any size — if at all. Gradually more growth appeared at a distance of 20–30 feet, and was normal beyond such a radius. This was suggestive of radiation injury and the wreck was monitored with caution. However, it showed no counts above background. Evidently the injury to algal growth was of some other sort — possibly a gasoline fire, or oil. It may be concluded that algal growth in the region is comparatively slow, since there had been no recolonization on the boat or nearby rocks. This gives good evidence on the *absence* of injury by the bomb and later events over the rest of the region. For, if there had been mass killing, recolonization would not have occurred, probably, in the course of one year.

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[1] The collaboration of Dr. P. M. Brooks and Dr. W. Gortner, in many of the experiments reported here, is gratefully acknowledged.

[2] During the Bikini Scientific Resurvey.

The northwestern spit of Bikini (A) and the adjacent coral heads just within the lagoon were the most radioactive regions readily available for the collection of living algae, while the easterly reef (B) and southern spit (C) of this island were relatively free from radiation. The reef off the Officers' Club (D) was also used somewhat. These chief regions were visited frequently for comparison and collection, the collected algae being brought in quickly, and with care to provide aeration and minimize warming. The specimens were used immediately, or when necessary, kept in running sea water for use on later days. Certain forms lived well *(Cladophoropsis, Valonia aegagropila, Dictyosphaeria, Rhipilia, Turbinaria, Halimeda opuntia* (?), *Lyngbya, Jania, Caulerpa serrulata);* others, e.g., *Udotea, Liagora, Caulerpa racemosa, Halimeda micronesica* (?), were very sensitive and could scarcely stand transport from the reef.

Study was made of at least one each of red, brown, and blue-green forms, as well as several green algae, from normal and radioactive regions; calcareous forms were also included when possible.

Preliminary counts made on the algae themselves (wet and dry) indicated only a low degree of radioactivity, scarcely above background in the majority of cases, even when the plants grew on fairly radioactive rock or sand. There was evidently little accumulation of radioactive fission products: this is consistent with the fact that most algae do not concentrate the heavier elements to any great degree (potassium and iodine being most markedly accumulated). Exceptions to this statement occur chiefly in *Halimeda,* which showed counts as high as 3 or 4 times background, even when well washed of adhering sand and silt (identification of this radioactivity is given later in this paper). Clumps of *Cladophoropsis* or *Valonia* (in ''pincushion'' form) frequently showed fairly high counts, which, however, dropped to low levels on washing the cells free of adhering sand and silt. Most other algae showed none.

In view of this low actual accumulation within the plant tissues, attention became focused on the possible effects of *external* radiation, from the substrate, or from silt or sand on the surfaces of cells. In many cases, this ran from 0.001 or 0.002 r/day up to 0.01 or even 0.06 r/day (approximate dosage values, based on portable Geiger counter monitor). Much of this radiation was absorbed by the algae, as shown by a large increase in count when the algal mass was stripped from the rock. While fairly low and within ''generally tolerated limits'' at the time of the resurvey, the intensity must have been much greater during the previous year, depending on the decay rates of the fission products. Any lasting effects of more intense radiation might therefore show up in alterations of function.

Among the physiological activities studies were: permeability, salt accumulation, bioelectric potential, pigment content, photosynthesis and respiration, enzyme activity, and calcification. Many of these are known to be sensitive to other radiations such as ultraviolet and X ray, as well as to poisons, higher temperature, etc. It was thought that one or more might be altered by the radioactivity of fission products either in or around the algal cells.

Cellular permeability and salt accumulation are often very sensitive indicators of injury. *Valonia,* a genus much used for such studies, occurs on the north point of Bikini where moderate radiation bombarded the cells constantly. Normally a high potassium content and absence of sulfate in the cell sap prevail; if injury had occurred, potassium chloride would have diffused out and SO_4^- ion entered. However no sulfate could be detected and the potassium content was high.

Dyes, such as methylene blue and cresyl blue, entered the cells of *Valonia* and *Dictyosphaeria* and became accumulated in the cell sap equally well in normal and in irradiated specimens. Also, the cells of *Microdictyon* (both normal and irradiated) showed continued shrinkage (plasmolysis) with 1 *M* glycerol added to sea water; while they both recovered within a few minutes after shrinking in ethylene glycol. This behavior indicates normal permeability to both water and nonelectrolytes. Evidently there has been no disturbance in this sensitive function.

The closely related *bioelectric potential* was also measured across the protoplasm of *Valonia forbesii* (the cells of which reach diameters of 1 cm or more). It was found to have the normal value (about 15 mv, outside negative) in cells collected both from stations A and B (radioactive and normal).

The plastid pigments (chlorophyll, carotenoids, and phycobilins) of algae are frequently very sensitive to injury, and change both in absolute and relative amounts due to irradiation, temperature change, or inorganic nutrition lack (especially iron deficiency). It was of great interest to see whether the Bikini algae were altered in their pigment complex. Extracts of weighed pieces of algae were made with methyl alcohol, and the absorption spectra determined in the Beckman spectrophotometer. Chromatographic separation of the pigments on sucrose or starch columns was also carried out. In neither green, brown, nor red algae was any alteration of pigment component ratios found which could be ascribed to radiation from fission products. Much greater dependence was found on solar radiation. Algae exposed to intense sunlight showed bleaching or yellowing of the upper parts — this was especially true of *Laurencia* and *Lyngbya* (a red and blue-green alga respectively). To determine whether the *absolute* amounts of the major pigment (chlorophyll) were altered, transmission percentage was read at the red absorption maximum (663 mμ). The values varied greatly from alga to alga on a dry-weight basis, but no consistent trend could be found between plants from normal and radioactive regions. *Cladophoropsis* from station A did have about twice as much chlorophyll as samples from station B; but this may have been ecological rather than radiological in origin, since the plants grow on much more exposed rocks and form denser cushions. Some 9 other genera were also studied, including red, brown, and green algae: no others showed this trend. As many showed increase as decrease, and many were nearly identical or within the normal variation.

In view of the absence of radiation effects on the chief photosynthetic pigment, it was of interest to study the over-all photosynthetic rate. This was done by measuring oxygen production, determined in solution in sea water by the Winkler method. The plants were usually put in 250-cm^3 flasks in a water bath over fluorescent lamps of an intensity about 200 ft c (one-fourth or one-fifth that of direct sunlight). Exposures of 1–6 hours were given, the latter being usually sufficient to saturate the sea water with oxygen. Rates were reduced to a common basis of cubic centimeters of oxygen/hour/g of wet weight of tissue. Values ran from 0.06 *(Valonia)* to 0.76 *(Bryopsis),* many algae showing about 0.1 cm^3/g/hour. No evidence of a trend in either direction could be ascertained. The number of cases of a higher rate in normal plants was balanced by those higher in irradiated ones, and the differences fell within the range of variation. Since photosynthesis is a complex process involving many successive energy transfers and enzyme reactions, injury at any stage might be reflected in a lower rate. No clear case of such was found. Evidently those plants which survive at all have an essentially normal photosynthetic mechanism. (If radiation had caused a critical break in the chain, such plants may have died out.)

Respiratory rate was also measured to compare with photosynthesis and make proper corrections for the latter. The method was the same except for keeping the plants dark. Rates between 0.0075 and 0.03 cm^3/g/hour were found in different algae, but just as with photosynthesis, no consistent difference could be found between plants from radioactive and normal regions.

Oxidative enzymes (oxidase and peroxidase activity) showed great variation between different genera of plants, but no effect ascribable to radiation.

This is not true, however, of the enzyme catalase. In at least three genera *(Halimeda, Udotea,* and *Cladophoropsis)* the release of oxygen by this enzyme was at a consistently higher rate in plants obtained from area A than from B. In *Halimeda* this ratio was 2- to 6-fold, averaging 3.7. In the two other genera it was at least twofold. (*Laurencia* and *Turbinaria* showed a smaller difference.) It is noteworthy that the three genera with the higher catalase activity were those exposed to the highest radioactivity or had the highest count themselves. No clear explanation is at hand for this effect but the suggestion is tentatively made that it is an ''adaptive enzyme'' response much like that known in other cases where a substrate change is made. It is known that hydrogen peroxide is formed by X rays and other radiations in water. It seems possible that this is occurring in these plants, the enzyme activity increasing to decompose the extra hydrogen peroxide (which is toxic). Another possibility is that a ''micronutrient'' necessary for the elaboration of catalase is provided among the fission products of the bomb (whether radioactive or not), or from sunken ships, etc. The role of elements in small traces in other plants suggests this. The *direct* catalytic effect of such metals is ruled out, since most of the catalase activity is destroyed on boiling; this indicates truly enzymic character.

Further experiment along these lines might be of value in the study of radiation effects.

Calcification is an important aspect of certain tropical algae, notably the ''corallines'' of the reef edge, but also of several green genera. The percentage of calcium carbonate was determined for various Bikini algae. No significant difference in calcium carbonate (64–66%) was found in *Halimeda tridens* collected from a radioactive region (A) and a normal one (B).

Identification of radioactive elements was attempted in samples of *Halimeda* collected on a dead coral head. This was in the direct path of the bomb surge wave and was killed either by radioactivity, high temperature, or silting. Some young coral colonies, and many young algae, were re-establishing themselves (though only in patches). These algae included *Halimeda, Udotea, Caulerpa, Avrainvillea, Microdictyon*, and *Pocockiella*. The older *Halimedas* were covered with silt and epiphytes, indicating poor condition of the plants.

These organisms received radiation ranging from 0.005 to 0.06 r per day, averaging 0.02–0.03. Such high persisting activity indicates an extremely high radiation intensity just after the blast. The *Halimeda* growing upon these rocks gave about 500 counts per minute. About 400 g of this alga were decalcified with hydrochloric acid, then washed well with water, which removed loose insoluble residues, epiphytes, etc. The acid solution was filtered, then precipitated with ammonia which threw down iron hydroxide and adsorbed substances. After filtration the solution was dried. The 4 fractions were roughly monitored for radioactivity, which ran as follows:

1. Decalcified plants (cellulose, etc.)	452 counts/minute
2. NH_4OH precipitate ($Fe[OH]_3$ etc.)	951 counts/minute
3. Suspended matter (debris, epiphytes)	6120 counts/minute
4. Soluble fraction (filtrate)	very low

Radiological and chemical properties ruled out zirconium as an important component, leaving cerium and praseodymium as the important radioactive constituents (nearly 100% of samples 1 and 3). Ruthenium-rhodium accounted for about 20–30% of sample 2 (ammonia precipitate).

DISCUSSION

The results, mostly negative, reported above are rather unsatisfactory, in being based on observation rather than experiment, and not well controlled. But they may have some value as a record of a unique occurrence, and in showing that marine plants tolerate low level, mixed β and γ radiation, for as long as a year without noticeable alteration of many normal somatic functions. If injurious genetic effects had occurred, they were still recessive, or perhaps were missed in the rapid, inadequate sampling performed in the time available. In any case they were not strikingly apparent. The only exception was the increased catalase activity, which might be of the nature of an enzymic adaptation, to hydrogen peroxide. This should be subjected to experimental test, since the peroxide hypothesis is an important one in explanation of radiation effects.

The Bikini results are further confirmation, if needed, that experiments with radioactive tracers are not unduly complicated by the effects of radiation itself on function, nor by the fission products resulting, if such radiation and products are of sufficiently low level.

CONCLUSIONS

The marine algae surviving at the most radioactive spot accessible on the Bikini Island reefs, one year after the atom bomb explosions, were abundant and like those of other, normal, regions nearby, both in appearance and in a variety of functions (pigment content, photosynthetic rate, respiratory rate, calcification, potassium content, permeability, bioelectric potential, and oxidase activity). Only in one function investigated, did algae from radioactive regions differ: their catalase activity was markedly enhanced, possibly in response to hydrogen peroxide produced by radiation. This suggests an ''adaptive enzyme'' effect.

THE EFFECTS OF RADIATIONS ON RESPIRATORY METABOLISM

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THREE FIGURES

It is generally agreed that the respiratory metabolism of living systems provides fairly accurate information about their over-all physiological state and that oxygen consumption and carbon dioxide output can be used, therefore, as sensitive indicators of the effects of various agencies on cells and tissues. In view of the profound effects which are exerted on living systems by X and other ionizing radiations, one might be led to expect that over-all metabolism, as reflected in gaseous exchange, would be rather promptly and significantly affected by radiation. Even a cursory survey of the literature shows, however, that in general such an expectation has not been realized. This may appear somewhat surprising, and especially so, since it has been shown that a number of enzymes which are known to participate in cellular respiration have reduced activity after irradiation *in vitro*.

In this review, several cases in which the respiration of cells, tissues, and embryos has been studied following X irradiation will be considered. I shall begin by presenting first some work on the effects of X rays on the respiratory metabolism of the grasshopper embryo, *Melanoplus differentialis*. The development of the grasshopper can be roughly divided into three phases. During the first of these, the so-called prediapause developmental period, the embryo increases in size through cell division and growth for approximately 20 days.[1] The second phase is one of developmental block or diapause. Cell division does not occur, and growth is at a standstill. Diapause may last for several weeks or months, but it can be terminated at will by the expedient of exposing eggs to low temperature for some time or by treating them briefly with xylene (Slifer, '48). After interruption of diapause, a postdiapause developmental period ensues, and this is terminated at the end of 17 or 18 days by the hatching of the young nymph.

Embryos immediately before diapause, during diapause, and shortly following diapause are morphologically identical, but physiologically they are quite different. Some of these differences are reflected in the changing respiratory rate of eggs during development (Boell, '35). During the prediapause period, respiration rises gradually to a peak on about the 20th day. With the onset of diapause, respiration declines to a basal level, and this is maintained with only minor fluctuations for the duration of the diapause. On interruption of diapause, respiration again rises coincidentally with growth and differentiation. The respiratory rate of developing eggs is from two to three times greater than that of diapause eggs, and the same difference in respiratory rate is exhibited by diapause and developing embryos which have been removed from the eggs and freed from yolk and extra embryonic membranes by dissection (Bodine and Boell, '36b, '37).

At the time that we undertook our work on the effects of X irradiation on the respiratory metabolism of grasshopper embryos, Evans ('34) had already established tolerance dosages for postdiapause eggs. A dose of less than 1000 r was without effect either on rate of development or on respiration. With a dose of 1000 r, he found that growth, development, and respiration were approximately normal but that the embryos failed to hatch. Dosages of 2000–6000 r exerted progressively greater effects on developmental rate.

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[1] Developmental time refers to a temperature of 25°C.

With 4000 r, for example, yolk engulfment did not occur, and development stopped. The respiratory rate of irradiated embryos was less than that of controls, but when corresponding stages of irradiated and control embryos were compared, it was uncertain whether or not respiration had been affected. Evans ('35) went on to show that resistance to X rays increased throughout development and that diapause embryos were the most resistant of all.

We were interested in determining whether X rays influenced the respiratory process, and, if so, whether the effects observed could be attributed to direct injury to the respiratory mechanism of the embryo or whether they were secondarily produced because of interference with development. Since the grasshopper undergoes a naturally occurring block which intervenes between two phases of active development, the effects of radiation could be compared on one and the same material during periods of cell division, growth, differentiation, and rapid metabolic turnover, on the one hand, with a period of developmental quiescence and minimal metabolism, on the other. Accordingly, we determined the immediate and delayed effects of X rays both on developing embryos and embryos in which development had been blocked (Boell, Ray, and Bodine, '37).

Some typical results on diapause eggs are shown in table 1. The procedure in these experiments involved irradiating a group of eggs with 2040 r, placing them at 25°C. together with control eggs, and then measuring the respiration of the eggs at intervals after irradiation. The table shows that the respiration of irradiated diapause eggs was completely normal for as long as 12 days. Actually, the respiration of diapause eggs was found consistently to be slightly higher than that of controls, but the difference between the two lots is probably without significance.

Observations were made, in addition, on the effects of X rays on embryos which had been removed from the eggs. The egg is a complex system, consisting of the embryo, extra embryonic membranes, and yolk. All these components respire

(Bodine and Boell, '36b), and it is possible that they might not all be affected in the same way or to the same extent by irradiation. The results show that the respiration of embryos isolated at various intervals after irradiation of the eggs is no different from that of control embryos (table 1).

Our results with diapause eggs were confirmed recently by Tahmisian ('49) who irradiated eggs with dosages of X rays ranging from 25,000 to 200,000 r. With only the strongest dosages was depression of respiration noted. Irradiation with lower doses was not without biological effects, however, for pycnotic nuclei appeared in the embryos of the irradiated eggs, and a phenomenon occurred, described by the author as *negative growth,* in which the embryos, although remaining alive, decreased progressively in size. Tahmisian concluded, therefore, that the anabolic phases of metabolism were much more susceptible to X-ray injury than the catabolic phases.

TABLE 1

The effect of X rays on respiration of grasshopper eggs and embryos during diapause

		DAYS AFTER IRRADIATION	RESPIRATION RATE [a]		
			Control	X-rayed	Per cent
Diapause respiration after 2040 r	Egg	1	9.7	10.1	104
		4	6.9	7.1	103
		7	6.4	6.6	103
		10	6.2	8.2	132
		12	5.1	5.4	106
	Embryo	5	6.4	6.9	108
		11	5.4	5.5	102

[a] Averages of triplicate determinations for eggs and duplicate determinations for embryos.

In our experiments with developing eggs and embryos, control and irradiated eggs were divided into two lots. One was kept at a constant temperature of 25°C. while the other was placed at 10°C. At the lower temperature, development is pretty effectively blocked so that the embryos remain at the same developmental stage for a considerable period of time. At intervals, respiration was determined, at 25°C., on both lots of eggs. A summary of the results obtained is contained in table 2. The respiratory rate of X-rayed eggs kept at 25°C. is definitely lower than that of control embryos at the same temperature, but that this is very likely the indirect result of retardation of development by X rays rather than a primary effect on the respiratory mechanism is indicated by the data for control and X-rayed eggs which were kept at 10°C. between measurements. In these experiments, we found that the respiratory rates for control and irradiated eggs were identical and constant throughout the 15 days over which respiratory measurements were made (table 2).

TABLE 2

Respiration of developing grasshopper eggs exposed to different environmental temperatures after irradiation

		DAYS AFTER IRRADIATION	RESPIRATION RATE [a]		
			Control	X-rayed	Per cent
Dosage — 2040 r	Temp. 25°C.	1	69.5	69.3	100
		4	81.2	76.6	94
		6	87.6	77.0	88
		8	79.0	73.0	93
		12	136.0	109.3	80
		15	159.5	133.0	83
	Temp. 10°C.	4	59.9	55.4	93
		6	64.8	62.8	97
		8	65.4	61.2	94
		12	65.4	64.0	98
		15	67.2	63.0	94
Dosage — 5000 r	Temp. 10°C.	1	61.5	64.5	105
		4	62.5	68.6	94
		6	62.1	62.0	100
		8	66.9	64.7	97
		12	67.2	63.9	95
		15	64.3	65.7	102

[a] Average of duplicate determinations.

Several experiments were performed on embryos which were irradiated *in vitro* after dissection from the eggs. In a typical case, the embryos were irradiated in buffered insect Ringer's solution. They were then washed in 4 changes of fresh solution before respiratory measurements were begun. The results of these experiments, summarized in table 3, show that irradiating embryos *in vitro* was without effect on respiration.

A number of studies (Bodine and Boell, '36a; Allen, '40) have shown that cytochrome oxidase activity, as measured *in vitro*, is essentially identical in prediapause, diapause, and postdiapause embryos of the same morphological stage of development. Yet evidence from other lines — e.g., insensitivity to cyanide and carbon monoxide (Bodine and Boell, '34a, b) — shows that the cytochrome system is not functioning, or at least not to capacity, in the diapause embryo. The activity of the cytochrome system can be increased in diapause embryos by treating them *in vitro* with various stimulating substances, among them methylene blue, and dinitrophenol or dinitrocresol (DNC) (Bodine and Boell, '38). These reagents bring about a marked stimulation of respiration, and the increased oxygen consumption is sensitive to cyanide and carbon monoxide. An experiment illustrating these

TABLE 3

Respiration of developing embryos after X irradiation in vitro with 2040 r

POSTDIAPAUSE STAGE	RESPIRATORY RATE	
	Control	X-rayed
1st day	38.3	37.9
1st day	39.2	37.9
1st day	38.3	40.3
Average	38.6	38.7
2nd day	42.7	42.5
2nd day	41.4	43.9
2nd day	41.5	42.0
Average	41.9	42.8

properties is shown in figure 1. The respiration which is stimulated by DNC is clearly depressed by carbon monoxide in the dark but not in the light. In figures 2 and 3, it can be seen that the respiratory mechanism which can be stimu-

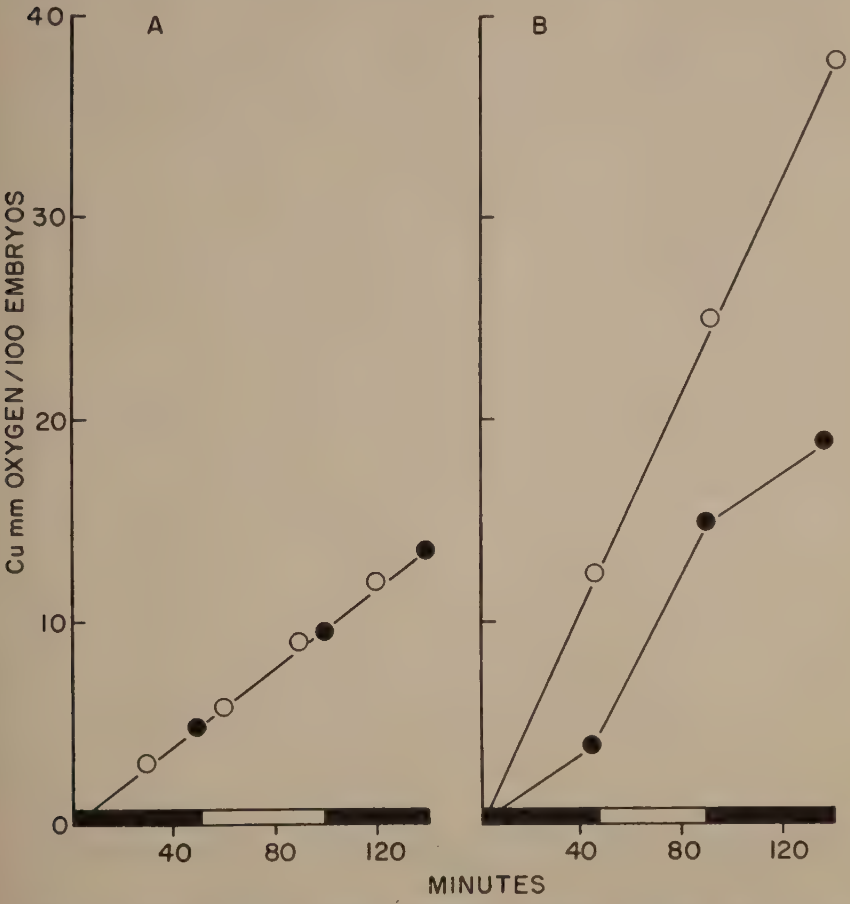

Fig. 1 Respiration of diapause grasshopper embryos. The left side of the figure shows the respiration of embryos in the presence of air and in a mixture of 95% carbon monoxide and 5% oxygen. The right side of the figure shows the stimulation of respiration by $2 \times 10^{-4} M$ 3,5-dinitro-*o*-cresol and the susceptibility of the stimulated respiration to CO. Periods in the dark and in the light are shown on the base line. Curves drawn from unpublished data of the author.

A — ○ = control	B — ○ = DNC
● = CO	● = DNC + CO

lated by DNC, presumably the cytochrome oxidase system, is not affected by X rays in either diapause or developing embryos. Tahmisian and Adamson ('50a) investigated the effect of X irradiation on the ability of homogenates of grasshopper embryos to oxidize hydroquinone in the presence of excess cytochrome c. The immediate effect of irradiation was a reduction of oxidase activity, but this returned to normal within two days after irradiation, and, as time went on, greatly exceeded the normal. The significance of this find-

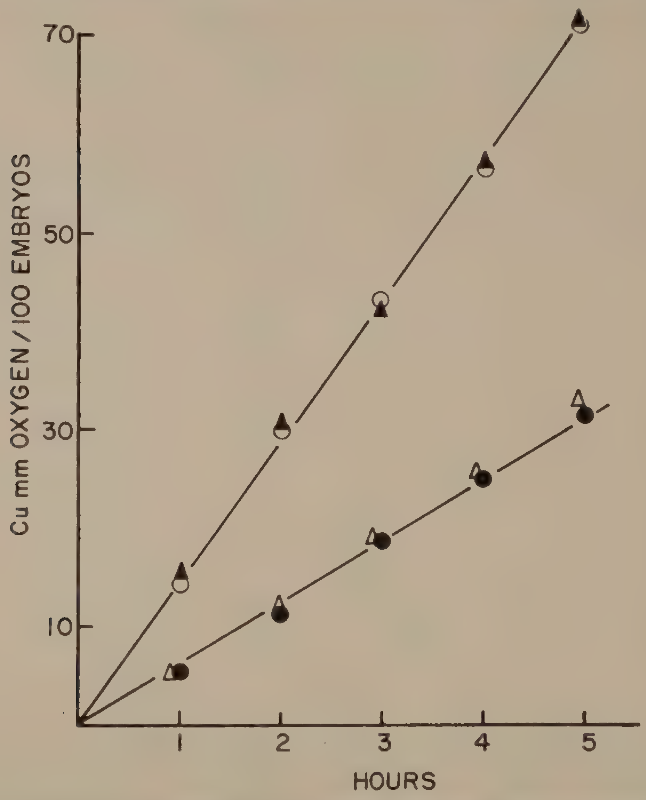

Fig. 2 Respiration of control and X-rayed diapause embryos in the presence of 2×10^{-4} *M* 3,5-dinitro-*o*-cresol. Respiratory measurements were made 5 days after irradiation with 2040 r (Boell, Ray, and Bodine, '37).

● = control   △ = X-rayed
○ = control + DNC   ▲ = X-rayed + DNC

ing is obscure, especially since the results were not completely reproduced when other substrates for the cytochrome system were used, and also because the increase in oxidase activity occurred during the time that the embryos were undergoing negative growth.

Recently, Tahmisian and Barron ('48) reported results on the respiration of grasshopper eggs after irradiation which differ somewhat from our own. They have claimed that the

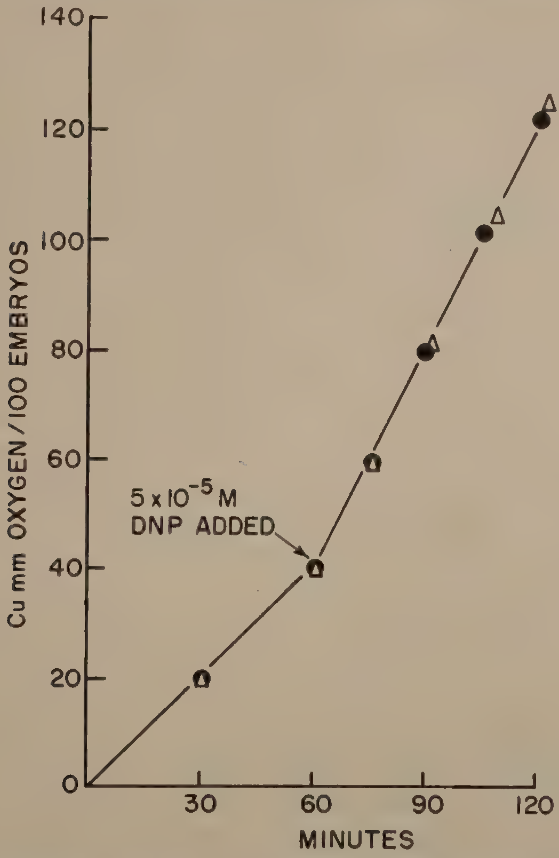

Fig. 3 Respiration of control and X-rayed developing embryos in the presence of $5 \times 10^{-4} M$ 2,4-dinitrophenol, 12 days after X-raying (Boell, Ray, and Bodine, '37).

● = control

△ = X-rayed, 2040 r units

respiration of grasshopper eggs is significantly depressed by as little as 10 r. The depression is of short duration, however, and respiration returns to normal levels within 5 hours. In our experiments, we generally did not begin respiratory measurements immediately after irradiating the eggs. Therefore, if a period of depressed respiration exists, it would have been overlooked. Except for the period immediately after irradiation, our findings appear to be confirmed by those of Tahmisian and Barron, and it seems clear that respiration in the grasshopper embryo, especially during diapause, is remarkably resistant to the effects of X rays.

A considerable body of recent evidence has been accumulated to show that X rays do not produce their effects altogether by direct action (target theory), but that a substantial proportion of the results of irradiation are brought about indirectly by products resulting from the ionization of water, such as H atoms, OH ions, O_2H radicals, and hydrogen peroxide (Fricke, '34; Dale, '40; Zirkle, '49). Indeed, a number of workers have concluded that irradiation effects can be duplicated, to some extent at least, by hydrogen peroxide. One might expect to find, therefore, that catalase would afford some protection against radiation damage in the cell. It is of some interest to recall, in relation to the resistance of diapause eggs to X rays, the work of Williams ('36) on the catalase activity of grasshopper eggs throughout development. It may be more than mere conincidence that the catalase content and resistance to radiation rise together during grasshopper development and that during diapause, when resistance of the embryos to X rays is greatest, the catalase content is much higher than in developing embryos of corresponding morphological stage.

Grasshopper embryos subjected to X radiation, in the dosages used, develop normally to hatching, although in some cases development is slower than in controls. The lethal effects of radiation are then expressed in the inability of the embryos to hatch. The data in table 4 illustrate this point. Three series of eggs are here represented. In the first, the eggs were given 2040 r and kept at 25°C. In the second, the eggs were given 2040 r, and then placed at 10°C. for 15 days before transfer to 25°C. for development. The third series was like the second except that the irradiation dosage was 5000 r. Unirradiated eggs were provided with the same temperature experience as the irradiated eggs. The percentage hatch in all three series was considerably lower from irradiated eggs than from controls, and, in the few cases where hatching did occur, the nymphs with one exception died in a very short time. In no case were the nymphs which hatched capable of completing the first molt. The table shows also that exposure of eggs to low temperature after irradiation did not permit recovery from the effects of X rays. This observation is somewhat similar to one reported recently by Patt and co-workers (Patt et al., '48; Patt and Swift, '48). These investigators irradiated frogs with 1000 to 9000 r and found that exposure to 10°C. greatly prolonged their survival. Following exposure to 3000 r, for example, there was only 7% survival in a group of animals kept at room temperature for 5 weeks, whereas, at 5°C. there was 100% survival during the same period of time. Qualitatively similar results were obtained after irradiation with 6000 and 9000 r. However, when such irradiated frogs were

TABLE 4

Effect of X irradiation on hatching of grasshopper eggs

DOSAGE AND TEMPERATURE	NUMBER OF EGGS	PERCENTAGE HATCHING	PERCENTAGE LIVING NYMPHS
2040 r, 25°C.	106 (C)	99	93
	112 (X)	20	0
2040 r, 10°, 25°C.	101 (C)	95	91
	102 (X)	9	1
5000 r, 10°, 25°C.	98 (C)	90	85
	99 (X)	8	0

(C) = control eggs.
(X) = irradiated eggs.

restored to room temperature, after a period in the cold, there was no appreciable difference in the percentage survival or the time course of deaths over animals kept continuously at room temperature. One can conclude that keeping grasshopper embryos or frogs at low temperatures simply delays the development of radiation damage. Recovery at low temperature does not occur.

A number of studies have been made on the effects of irradiation on the respiration of sea urchin gametes and fertilized eggs. It is a well-known fact that irradiation of gametes results in delayed mitosis after fertilization and in the production of developmental abnormalities of various kinds (see the reviews of Butler, '36; and Giese, '47). Chesley ('34) subjected *Arbacia* eggs, both before and after fertilization, to dosages of X rays as high as 43,000 r and reported that respiration was unaffected by this treatment. Neither was the acceleratory effect of methylene blue on egg respiration influenced by irradiation. Evans ('40) showed that irradiation of either eggs or sperm did not suppress the rapid increase in respiration which normally occurs when they are brought together in fertilization. During subsequent development, the oxygen uptake of control eggs seemed to be higher than that of irradiated samples, but this was probably the indirect result of developmental lag in the irradiated eggs. It appears from further work of Evans ('47) that the effects of irradiation of sperm suspensions are due primarily to the radiation products of water. Heavily irradiated sea water (100,000 r) had a deleterious action on *Arbacia* sperm which were added to it. Such spermatozoa had reduced survival, and eggs fertilized by the spermatozoa exhibited delayed first cleavage. Evans concluded that the chief, if not the only, agent responsible for the radiation effects was hydrogen peroxide. The importance of peroxide in the irradiated medium was shown by the similarity of its action when added to unirradiated sea water, by chemical tests, and by the protective action afforded by catalase. The situation is not completely clear, however, since it was claimed that boiled catalase was also effective in providing protection against radiation damage.

Barron, Gasvoda, and Flood ('49) have recently investigated the effects of X rays on the respiration of sea urchin sperm. Sperm suspensions, made up in a dilution of 1:200, were found to have reduced respiration after irradiation. With 100 r, a depression of oxygen uptake of 10% was noted, and this was found to increase with increasing dosage of X rays to 66% depression at 20,000 r. Succinate and acetate stimulate the respiration of sea urchin sperm suspensions, but the stimulated respiration could be completely abolished with 100 r. According to Barron and his associates respiration of sperm is inhibited on the addition of irradiated sea water, but the effects in this case could not be attributed to hydrogen peroxide, for, although peroxide inhibits sperm respiration, it does so only in concentrations of 0.001 *M* or stronger. With weak concentrations, corresponding in strength to those produced when sea water is irradiated, respiratory stimulation results. Moreover, Barron and his co-workers show that catalase is present in sea urchin sperm and claim that if hydrogen peroxide is produced on X irradiation of water it would be broken down by catalase. If this be true, it is difficult to see why the addition of hydrogen peroxide to sperm suspensions should then be effective in stimulating respiration.

In a second paper, Barron, Flood, and Gasvoda ('49) reported results of a further investigation on the effects of irradiated sea water on sperm respiration. Sea water which had been irradiated with 100,000 or 200,000 r reduced the respiration of sperm suspensions 38 and 60% respectively. It was concluded that the action in this case was not due to hydrogen peroxide formation since none was detectable chemically, and since catalase afforded no protection. It was postulated, therefore, that stable, organic peroxides were produced on irradiating organic matter in the sea water and that these were responsible for the effects observed.

The question arises as to whether the metabolic level which prevails in the cell or organism at the time of irradiation is in any way correlated with its radiosensitivity. A number of suggestions in the literature might lead one to conclude that radiation sensitivity is lower in cells or tissues when metabolism is depressed. Thus, as early as 1927, Strangeways and Fell found that, if tissues from irradiated chick embryos were placed at 0°C. for 5 hours prior to culturing them at 38°C., good growth was obtained. By contrast, tissues from embryos which had been similarly irradiated but allowed to incubate for 5 hours did not grow when subsequently placed under conditions for tissue culture. Evans, Goodrich, and Slaughter ('41) were able to show that the radiosensitivity of newborn rats varied directly with the temperature at which they were irradiated between 0° and 40°C. The difference in effect produced by a given dosage over this temperature range was equivalent to a dosage range of 0–2500 r at 25°C. Newborn rats are poikilothermous, so that their metabolism is greatly depressed at low temperatures.

With adult mice the effect of low temperature on radiation sensitivity is exactly opposite to that reported by Evans and his co-workers. Mice subjected to a lethal dose of approximately 500 r were found by Smith, Highman, Mitchell, and Blount ('49) to survive somewhat better if subjected to a temperature of 30°C. after radiation than when exposed to 10°C. These authors cite data of others which show that total metabolism of mice is elevated at low temperature. Thus, in experiments in which the animals were unable to huddle together, 69% in the 10°-room died as compared with 39% in the 30°-room, and the authors conclude that "resistance to lethal X-ray effects is lowered in a cold environment when the oxidative metabolism of mice is elevated." The observations of Patt and Swift ('48) and of Patt, Swift, and Tyree ('48), on the radiation sensitivity of frogs at different temperatures, have already been mentioned. It will be recalled that sensitivity to radiation effects is not revealed as long as frogs are kept at low temperatures, but, on returning them to room temperature, the mortality curves are not significantly different from those of animals kept constantly at warmer temperature. Patt concludes, therefore, that the metabolic level of frogs during and after irradiation does not materially affect their radiosensitivity.

Recent experiments by Bacq and co-workers ('50) have shown that injection of sodium cyanide into mice prior to irradiation with 500 to 600 r protects the recipients from the lethal effects of X rays. Bacq believes that the amount of cyanide given was insufficient to reduce disulfide linkages and that reduced sensitivity to radiations in the presence of cyanide is due either to depression of metabolism or to the inactivation of catalase. Bacq's observations have apparently not been confirmed, for Dowdy et al. ('50) were unable to show that cyanide provided rats with any protection against radiation damage.

A number of workers have shown that radiosensitivity is lessened with reduction or complete absence of the oxygen supply to the cells or tissues during irradiation. The survival of various bacteria after a dose of 60,000 r is 1000 to 100,000 times better on irradiation in nitrogen than in oxygen (Hollaender et al., '51). Thoday and Read ('47) irradiated root tips of *Vicia faba* in nitrogen, air, and pure oxygen and found, with a given X-ray dosage, that growth was interfered with less after irradiation in nitrogen than in either of the other two gaseous media. Similarly, chromosome aberrations were much more numerous in oxygen than in nitrogen. Giles and Riley ('49, '50) and Giles and Beatty ('50) obtained similar results with *Tradescantia* microspores and were able to show, in addition, that the effect of oxygen during irradiation was to enhance the breakage of chromosomes rather than to influence the recombination of their broken ends.

Animal cells, likewise, are less susceptible to irradiation damage in the absence of oxygen. Evans and his co-workers ('42) have reported that by ligating the tail so as to block circulation or by taping the mouth so that breathing cannot occur, the radiosensitivity of rats is greatly reduced. Furthermore, Baker and Sgourakis ('50) have shown that the number of lethal mutations in the X chromosome of *Drosophila* was much greater on irradiating in oxygen than in nitrogen. These workers also demonstrated that the effect in oxygen was greater at 2° than at 27°C., presumably because of the increased solubility of oxygen in the tissues at the lower temperature. With the grasshopper embryo, Tahmisian and Adamson ('50b) reported that negative growth, which normally follows irradiation of diapause embryos with 25,000 r, was prevented when the embryos were irradiated in a gas mixture consisting of 5% carbon dioxide in nitrogen. Such embryos, in contrast to those irradiated in air, developed to hatching after the termination of the diapause. Even in mammals, the enhancement of radiation damage by oxygen apparently takes place, for Dowdy, Bennett, and Chastain ('50) found that when rats were exposed to only 5% oxygen the acute effects of irradiation were only one-half as severe as when they were exposed to air.

At first glance, it might seem that the numerous reports on the reduction of X-ray effects in the absence of oxygen lend support to the view of certain of the investigators cited that radiosensitivity varies directly with metabolic rate. However, an alternative and more widely accepted explanation of the potentiation of X-ray effects by oxygen lies in the participation of oxygen in the production of active substances from water during the irradiation process.

CONCLUSIONS

It is clear from the foregoing review that complete accord does not exist as to the effect of X rays on respiratory exchange or on the importance of metabolic level in determining radiosensitivity. It is, therefore, difficult to draw completely satisfactory conclusions from the data presented. It has been shown by Dale ('40, '47) and Barron et al. ('49), to cite only two investigators who have worked in this field, that certain enzymes, including a number which are concerned in oxidative metabolism — adenosinetriphosphatase, and phosphoglyceraldehyde and succinic dehydrogenases — are inactivated after irradiation *in vitro*. Barron ('48) has also shown that respiration of tissue slices from a number of organs was depressed by irradiation, especially when the tissues were metabolizing substrates for sulfhydryl-containing enzymes. Barron's studies demonstrate that those enzymes whose activity depends upon sulfhydryl groups are particularly susceptible to radiation injury. He has concluded that enzyme inactivation in these cases is due to the oxidation of sulfhydryl groups in the active centers of the enzymes by various oxidizing radicals, such as OH and O_2H, or by hydrogen peroxide, which are products of the ionization of water. In certain cases, he has been able to show that partial recovery of enzyme activity after irradiation could be achieved by treating enzyme solutions with glutathione so as to restore the sulfhydryl groups. Parenthetically, the observations of Patt and his co-workers (Patt et al., '49, '50; Smith et al., '50) are of interest in showing that cysteine, administered intravenously to rats as late as 5 minutes before irradiation, afforded protection against an otherwise lethal dose of X rays. The protective action of cysteine quite probably lay in its ability to prevent the oxidation of certain cellular components by products resulting from X rays. Similar protection of *Escherichia coli* by sulfur-containing and also certain sulfur-free compounds has been reported by Burnett, Stapleton, and Hollaender ('51); and Hall ('51) has found that methylene blue injected prior to irradiation affords protection to mice.

In view of the demonstrated susceptibility of enzymes to radiation, it is somewhat surprising to discover that the respiratory process which is mediated by these enzymes in the intact cell is not more adversely affected by radiation. A number of suggestions may be offered in partial explanation of this state of affairs. In the first place, it is quite conceivable that the effects of radiation on enzymes are dissimilar *in vivo* and *in vitro*. In this connection, it may be mentioned that Holmes ('39) showed that X rays applied to tumor slices markedly inhibited glycolysis of glucose, and she concluded that the primary effect of radiation was to bring about inhibition of hexokinase activity, for the ability of the tumor tissue to break down hexosephosphate was unimpaired by irradiation. On the other hand, Barron, Dickman, Muntz, and Singer ('49) found that irradiation of solutions of crystalline hexokinase from yeast with X rays reduced enzyme activity only slightly. Among a number of enzymes studied by Barron which depend for their activity upon the presence of sulfhydryl groups, hexokinase was one of the most resistant to X-ray inactivation. Secondly, it is possible that in some cases enzymes are protected from radiation damage in the intact cell by the presence of nonenzymatic proteins, by catalase, or even by contact with the substrates whose breakdown they normally catalyze. It is likewise highly probable that many of the oxidative enzymes are present in the cell in such excess that they are not rate-limiting factors in respiration. A considerable degree of enzyme inactivation might occur, therefore, without resulting in any demonstrable effect on total respiratory exchange. Finally, it may be suggested that, although certain enzymes are not susceptible to X irradiation, their synthesis may be affected. Thus the effect of irradiating intact cells will express itself in terms of reduced enzyme and respiratory activity only after a latent period. Such an explanation seems applicable especially to the embryo in which the deleterious after effects of radiation on respiration seem to be correlated with damage to the synthetic machinery of the developing system.

Respiration is an over-all expression of reactions by which energy is liberated in cells and tissues. Since the respiratory process involves a multiplicity of enzyme-catalyzed steps and events, the type of result which one obtains on irradiating intact living systems will depend upon which steps are affected and to what extent damage has been done. In any case, the demonstration that the effects of radiation on biological systems are, in large measure, the indirect results of the ionization products of water and that enzymes are susceptible to such substances transports the problem of radiation effects, to a large extent, from the realm of biophysics into biochemistry. Future advances along this line should be profitable and exciting.

DISCUSSION

WILLIE W. SMITH: We have found that feeding thyroid elevates the oxygen consumption of mice approximately 50% above the normal. Such thyroid-fed mice are much more susceptible to X rays, as far as lethality is concerned, than normal. Irradiation, however, has no effect on oxygen consumption.

BOELL: Do you attribute the greater X-ray susceptibility of the thyroid-fed mice to their increased metabolism?

SMITH: We have no other conclusion at the present time. There is an increased metabolism, of course, with thyroid feeding or after dinitrophenol administration, and there is an increased mortality after irradiation in both cases.

BOELL: It is possible that, in special cases, radiosensitivity and metabolic level appear to be correlated, but the available data are by no means in agreement on this point. It would seem, therefore, that alternative explanations of the increased radiosensitivity of animals with elevated metabolic rate should be sought.

PATT: I should like to add that I think it is very important in all considerations of the effect of metabolic rate on radiosensitivity to distinguish the events which presumably take place during the irradiation from those which take place after exposure. I think a careful search of the literature will show that a change in metabolic rate or a change in temperature *during* irradiation does not influence the sensitivity to radiation. On the other hand, changes in temperature or metabolic rate *after* irradiation do have some influence. The influence may be simply a prolongation of the latent period or a slowing up of the development of injury.

In the experiments of Evans, to which you referred, I think it is likely that the effect of temperature in the newborn animal might very well be explained on the basis of reduced circulation which then would affect the oxygen tension of the irradiated tissues.

BOELL: I think it is highly probable that, in the case of newborn rats studied by Evans, interference with circulation, and the resulting reduced tissue oxygen tension, had a very profound influence on the results. Evans did make measurements of heart rate as affected by temperature and showed that heart beat went down from several hundred per minute to a rate of 14 or 15 a minute.

PATT: I might make one more comment on the effect of metabolic rate on radiosensitivity in mammals. Since it is not very feasible to change the body temperature of a mammal, we attempted to alter the rate of metabolism by severe exercise. So we actually made mice swim for 15-, 30- and 60-minute intervals prior to irradiation. Neither the half hour nor the hour of exercise prior to irradiation, which presumably elevated the metabolic rate, had any influence whatsoever upon the sensitivity of the animals.

D. E. SMITH: As a pharmacologist, I should like to point out the parallelism between the effect of radiation of the whole animal and the effects of certain toxic drugs. Death in both cases may be due to effects on specific organs rather than on the animal as a whole. If such an organ represents a relatively small part of the entire animal, a significant change in over-all metabolism following irradiation need not be expected.

KATZ: In this connection, it is interesting to observe that susceptibility to X rays in the rat can be decreased by protecting the adrenals with a lead shield.

HOLLAENDER: Doctor Boell mentioned some of our experiments on the effect of certain chemicals. It might be interesting to mention here that a group in our laboratory has found that there are 4 groups of compounds which give significant protection to bacteria against radiation damage.

Cysteine and BAL are typical for group 1. Then group 2, consisting of OH compounds like glycerine, propylene glycol, triethylene glycol, as well as the simple alcohols such as methanol, ethanol, and propanol, will protect very well. Reducing compounds (group 3), like sodium hydrosulfite, are excellent protectors in extremely low concentrations. Finally, in group 4, carboxylic acids, e.g., formic or succinic, afford excellent protection if the bacteria are incubated in the presence of these compounds for 30 minutes before irradiation.

BOELL: I think your observations are extremely interesting. To me they suggest that it is very likely that the components in the cell which are normally susceptible to irradiation — and these obviously include many more than those classified as sulfhydryl-containing enzymes — are insulated either physically or chemically by the compounds you have studied so that their active groups are protected from the deleterious effects of irradiation. I am reminded of this because of the similarity between the situation you describe and certain cases of enzyme inhibition. A particular case in point concerns the inhibition of cholinesterase activity by DFP. DFP is a powerful inhibitor of cholinesterase, but its effect is practically nil if the administration of DFP to the enzyme follows the addition of acetylcholine. In other words, the enzyme, by being combined with its normal substrate, is protected from DFP. Likewise, if one takes an intact animal and injects it first with a so-called reversible inhibitor of cholinesterase, such as eserine, and then treats it with DFP, one observes that eserine exerts a strong protective action. In similar manner, it seems possible that either naturally occurring or artificially administered substances may tie up the active centers of intracellular enzymes and thus protect them from radiation damage.

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THE INTERPRETATION OF CHEMICAL ANALYSES AND ENZYME DETERMINATIONS ON ISOLATED CELL COMPONENTS

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INTRODUCTION

Work on fractionation of cell components by physicochemical procedures, with subsequent analysis of the isolated components for various enzymes, proteins, nucleic acids, etc., is appearing in the literature at an ever-increasing rate. In the majority of the cases, a single method is used for separating the components and the assumption is made that the isolated component has neither gained nor lost material during the process of isolation. This talk is intended to show that such an assumption is not necessarily correct, and to outline procedures for determining what loss or gain in substance may occur in cell components while they are being isolated. Some of the results already recorded in the literature will then be re-examined in the light of the ideas that are developed.

BRIEF REVIEW OF PHYSICOCHEMICAL METHODS COMMONLY USED IN ISOLATING CELL COMPONENTS

References to the general subjects included under this heading will be found in Sumner and Myrbäck ('50). The cell components ordinarily prepared in reasonably pure condition by physcicochemical methods are the mitochondria, the microsomes, nuclei, and chromosomes, or chromatin threads.

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Certain other material, such as particulate glycogen and melanin granules, will not be considered at this time.

The most commonly used method for obtaining mitochondria is one developed by Hogeboom, Schneider, and Palade ('48), in which 0.88 *M* sucrose (strongly hypertonic) is used as homogenizing medium. The Potter-Elvehjem ground-glass homogenizer is usually employed to break the cells. The Waring Blendor can be used under some conditions to prepare mitochondria of good microscopic appearance, but using sucrose as suspending medium without pH adjustment, this type of homogenization is said to result in loss or destruction of some enzyme systems normally present in mitochondria (Schneider and Hogeboom, '51).

It might be added for the benefit of those who insist upon universal use of the Potter-Elvehjem homogenizer, that it is possible to regulate the speed of the Blendor over such wide range that the speed should be taken into account and specified by anyone claiming that it is or is not useful for a certain procedure. Following homogenization, the mitochondria are isolated by repeated differential centrifugation in the cold using more 0.88 *M* sucrose as suspending medium.

The microscopic appearance of the particles isolated in the hypertonic sucrose is remarkably similar to that of mitochondria in intact cells, at least in the case of liver cells, and there can be little doubt that the method produces good yields of isolated mitochondria of a high degree of purity.

Lately the use of isotonic sucrose has been recommended for preparing mitochondria (Hogeboom and Schneider, '50). This suspending medium produces rounded mitochondria which, however, contain at least one enzyme system that cannot be demonstrated in mitochondria prepared using 0.88 *M* (hypertonic) sucrose, namely, the enzyme system needed to synthesize *p*-amino hippuric acid (Kielly and Schneider, '50). Also, octanoate oxidation is said to be weaker with mitochondria isolated in hypertonic sucrose than with mitochondria isolated in isotonic sucrose solution (Schneider, '48). It is therefore clear that, in working with isolated cell particulates, "cytological integrity" is not a criterion of biochemical integrity even when apparently mild conditions prevail during the isolation procedure.

In the past, mitochondria have also been obtained using water or physiological saline as suspending medium. However, the use of water, at least, causes difficulty in allowing the escape of certain material (cytochrome c) from the mitochondria (Schneider et al., '48), and difficulty with agglutination is likely to be encountered using isotonic saline solution (Schneider, '48).

Microsomes can be prepared from the supernatant fluid remaining after centrifuging down the mitochondria. If hypertonic sucrose is used, dilution to isotonic strength is advisable, and even then considerable speeds are required (18,000 g) with centrifugation for as long as an hour in some cases. A centrifuge of fairly high speed such as the Servall or an ordinary centrifuge with a high-speed attachment is necessary for sedimenting the microsomes. If such centrifugal speeds are not available, microsomes which are probably suitable for nucleic acid studies are said to be obtainable by lowering the pH to about 4.5, whereupon the microsomes aggregate and are easily removed as a precipitate.

The microsomes do not seem to be of uniform particle size, since various fractions can be obtained according to the speed and time of centrifugation (Barnum and Huseby, '48). Purification of the microsome fraction is accomplished by recentrifuging in more of the suspending medium. The centrifuged material packs as a translucent jelly-like pellet in the bottom of the centrifuge tubes. The microsome fraction has been prepared in hypertonic and isotonic sucrose, physiological saline, and distilled water, but other media probably could also be used.

Cell nuclei have been prepared by a number of methods. Ground-glass homogenizers, Waring Blendors, and special high-speed blenders have been used to break the cells. Homogenizing media which have been employed include hypertonic sucrose, isotonic sucrose, distilled water with varying amounts of citric acid added, distilled water with calcium chloride added, and glycol solutions (Sumner and Myrbäck, '50). In addition, a method was devised by Behrens ('39) which avoided the use of aqueous media. This will be discussed briefly later.

In preparing cell nuclei in aqueous media, the first problem is to break the cells and disperse the cytoplasm finely without destroying the nuclei. If the cells are not almost completely disrupted, removal of whole cells usually proves to be very difficult. With the proper adjustment of pH, it is possible to break a very large proportion of liver, kidney, or pancreas cells without breaking a large proportion of nuclei, using the Waring Blendor running at the proper speed. In the case of some tissues, especially certain tumors, the mere breaking of the cell wall does not suffice to disperse the cytoplasm, which is very coherent and may adhere firmly to the nuclei in fragments. In such instances special methods, such as use of the colloid mill or the sonic oscillator, are necessary to produce even approximately pure nuclei. Fiber often is very troublesome and may have to be removed by special methods, such as forcing whole tissue through a fine screen under high pressure or straining the homogenate through cotton flannel.

Once the nuclei are liberated, there is danger of their complete disintegration. Such disintegration can occur partly as the result of mechanical action of the homogenizer and partly as the result of autolysis. Liver cells, for instance, contain proteolytic enzymes which operate at or slightly above neutrality and which appear to cause nuclear disintegration at such pH values. Although the nuclei themselves contain proteolytic enzymes (Miller and Dounce, unpublished research), it may be that the cytoplasmic enzymes are even more dangerous, since isolated nuclei do not autolyze as rapidly as nuclei suspended in the cytoplasmic supernatant.

To prevent disintegration of the liberated nuclei one can work with hypertonic sucrose using the Potter-Elvehjem homogenizer, in which case the nuclei are somewhat more stable than when distilled water is used as homogenizing medium. Still greater stability results, however, when the pH is lowered to 6 with a small quantity of dilute citric acid; or when a small amount of calcium chloride is added to the system in distilled water without lowering the pH (Schneider and Petermann, '50).

If the pH is reduced to less than 6 the cytoplasm agglutinates to such an extent that subsequent isolation of nuclei is difficult or impossible. The addition of appreciable quantities of salt favors this agglutination. When the pH is 4 or lower, the cytoplasm no longer agglutinates but most enzymes have been destroyed.

Some investigators insist on the use of 3 to 5% citric acid as medium in isolating nuclei. This leads to a very clean preparation judging by microscopic appearance, but the nuclei are useless for enzyme studies and, in addition, appear to have lost much pentose nucleic acid (PNA) and protein.

Nuclei are isolated from whatever homogenizing medium is used by differential centrifugation *in the cold.* If the centrifugation is not carried out in the cold (ca. 0°C.) certain enzymes like catalase may be extracted or inactivated.

In the method of Behrens, the tissue is quickly frozen and lyophilized and the dried material is ground in a ball mill. Nuclei can be separated by employing specific gravity flotation combined with differential sedimentation using organic solvent mixtures such as benzene-carbon tetrachloride for suspending medium. The procedure is, however, tedious and somewhat laborious. It is doubtful whether the nuclei are of as high purity as those prepared in aqueous media, but losses of water-soluble material from them cannot occur during the isolation procedure.

Chromosomes or chromatin threads are isolated from whole tissue which has been homogenized in physiological saline or sometimes in sucrose solutions. In the past it has not been possible to obtain the chromosomes from previously isolated cell nuclei, since rupturing the nuclei generally would break up the chromosomes as well. Hence the original homogenate, made in such a way as to rupture the nuclei, was subjected to careful differential centrifugation in order to segregate and finally isolate the chromosomes. We have observed, however, that previously isolated nuclei can be broken by means of a commercial sonic oscillator in such a way as to liberate considerable quantities of apparently intact chromosomes, together with nucleoli, plus some very finely divided material. Whether much would be accomplished by isolation of these chromosomes seems somewhat doubtful, however, since no protein is made soluble by this procedure. Attacks which have been made recently on the concept that isolated chromosomes are in fact what the name implies (Schneider and Hogeboom, '51; Lamb, '49, '50) are in our opinion without foundation.

Particulate glycogen and melanin granules can be isolated from liver cells by homogenization followed by differential centrifugation, but will not be considered further. The condensed Golgi apparatus of the histologists recently has been shown apparently to be an artifact (Palade and Claude, '41, '49), but its particulate precursor might conceivably be isolated by physicochemical means (Beams and King, '34). Isolation of nucleoli has not yet been accomplished, although this might conceivably be done.

EVALUATION OF CHEMICAL ANALYSES AND ENZYME STUDIES OF ISOLATED CELL COMPONENTS

The question now arises as to whether chemical and enzyme analyses of isolated cell components are to be accepted at their face values or whether considerable interpretation of the results is necessary in order to arrive at valid conclusions. From material already at hand it seems apparent that the latter situation prevails. We shall consider first the mitochondria, then the nuclei and chromosomes, and finally the microsomes.

Mitochondria. In the early work on isolation of cell cytoplasmic granules, mitochondria were isolated from homogenates made with distilled water or isotonic saline solutions.

When distilled water was used as homogenizing medium, cytochrome c appeared in loosely bound form both in the mitochondrial and in the microsome fractions. The portion found in the mitochondrial fraction was removable by washing with saline and was not fully effective in reacting in the cytochrome oxidase system. This situation did not occur when saline was used as homogenizing medium, since the cytochrome c remained in the mitochondria and was fully active enzymically. This shows immediately the importance of what type of medium is used for homogenization. Even in isotonic saline solution the mitochondria become swollen and rounded, and it seems possible that some protein might be extracted by the saline (Sumner and Myrbäck, '50).

Lehninger and Kennedy ('48) found that the fatty acid oxidase system of liver cell mitochondria is not active unless neutral salt such as sodium chloride or potassium chloride is present. This may be related to the physical integrity of the mitochondria.

When hypertonic sucrose was introduced as a medium for obtaining isolated mitochondria, it seemed to be taken more or less for granted that this medium was to become standard for preparing mitochondria. However, from what has already been stated, it is apparent that hypertonic (0.88 *M*) sucrose is not an ideal medium, and it is quite possible that isotonic sucrose will not prove to be ideal, in the sense of being applicable to the preparation of mitochondria suitable for any kind of chemical or enzyme analysis.

Therefore, considerable investigation should be carried out to ascertain the effects of various solutions in possibly extracting protein and enzymes from the mitochondria or in inactivating enzymes. Owing to the large surface presented by mitochondria, the possibility of adsorption also should be considered much more seriously than in the past. Certain important enzyme systems such as the cytochrome oxidase system and succinic dehydrogenase are, however, so difficult to make soluble that it seems certain that they are an intrinsic part of the mitochondria.

Nuclei and chromosomes. Where specific references are not included in this section, the results are taken from previously unpublished work of A. L. Dounce and Shirley R. Barnett. There are now available so many different homogenizing media for isolating cell nuclei that we can begin to draw certain conclusions about the effect of such factors as salt concentration and pH on the chemical composition of isolated cell nuclei, and it may be possible eventually to extrapolate back to conditions obtaining in the intact cell. It has been found, for instance, that nuclei isolated in isotonic saline solution at pH 7, with calcium chloride added to prevent autolytic destruction (Schneider and Petermann, '50) contain a higher amount of desoxyribonucleic acid (DNA) than nuclei isolated at pH 6 or 4 in distilled water (Dounce, '43; Dounce and Beyer, '48; Dounce, '50). This probably indicates a loss in protein, an idea which is strengthened by the fact that considerable protein can be extracted by isotonic saline from nuclei prepared at pH 6 in very dilute citric acid solution. Nuclei prepared in isotonic sucrose solution at pH 6 have the same amount or only a slightly smaller amount of DNA than nuclei prepared in dilute citric acid at pH 6, and hence presumably have about the same protein content as the latter.

How can one proceed further to find out what losses are changing the composition of the nuclei prepared in different homogenizing media? This might be done by analyzing nuclei prepared in different media for DNA content per dry weight and DNA content per average cell nucleus as follows: ''Standard'' nuclei are prepared in distilled water with sufficient citric acid added to adjust the pH to 4, since desoxyribonucleoprotein is insoluble at this pH. ''Standard'' nuclei prepared at a higher pH using 0.9% sodium chloride in the presence of calcium chloride as homogenizing medium might also be used, although the latter are somewhat more difficult to obtain. The amount of DNA per cell nucleus is then calculated from the standard nuclei, which have lost no DNA, and also for nuclei prepared by other methods. The counting procedures which must, of course, be used in this work unfortunately limit the accuracy of the experiments to some extent. If no appreciable quantity of DNA is lost in any of the nuclei, subsequent determination of the percentage of DNA on a dry weight basis will then reflect changes in protein (and to a minor extent lipid) concentration in the various nuclei. If a known amount of DNA has been lost by some nuclei, corrections can be made in calculating the amount of protein gained or lost. The results will be on a relative basis only and will show only the minimal amount of protein to be expected in the cell nucleus. Adsorption presumably should not influence the results materially, because of the relatively large size of nuclei. The absolute amount of protein present in the nucleus then might be estimated using the Behrens type nuclei (Behrens, '39) which, however, may not be as pure as those prepared in aqueous media; or by use of the microspectrograph, as is done by Pollister and collaborators (Pollister and Ris, '47). The latter method presumably is by no means exact. The above procedures constitute a systematic approach to the problem of what materials are lost or gained by cell nuclei during their isolation. An additional procedure would be to determine *nitrogen per nucleus* using nuclei obtained in different media.

It seems unwarranted to the writer to make the a priori assumption that the nuclear membrane can prevent loss of protein from cell nuclei. For this reason remarks concerning the necessity of obtaining nuclei of the same microscopic appearance as those in the cell (Schneider and Hogeboom, '51) seem irrelevant. It has already been demonstrated by the work of Hogeboom and Schneider ('51) that, in the case of mitochondria, a microscopic appearance similar to that of mitochondria in the intact cell is not a criterion of biochemical integrity. In the case of cell nuclei, it is known that the presence of electrolytes in amounts giving solutions approximately isotonic with plasma, or the lowering of the pH tend to cause condensation of chromatin material due apparently to slight dehydration. It is also known that sucrose solutions tend to cause hydration and swelling of the chromatin. It is meaningless, however, to try on a priori grounds to connect the state of hydration of the chromatin material with biochemical integrity, since the hydration of chromatin is said to be reversible (Ris and Mirsky, '49).

It has been known for some time that over 50% of the protein of an unbroken liver cell can be washed out by means of physiological saline solution without altering the main outlines of cell structure (Mirsky and Pollister, '42), and the same sort of thing may well be true of the nucleus, since isotonic saline extracts considerable protein from nuclei prepared at pH 6 with very dilute citric acid. The nuclear membrane is said to be permeable to fairly large dye molecules in the living cell. In the case of certain amoebas, the permeability of the nuclear membrane to dyes seems to be much greater than the permeability of the cell membrane (Clark, '43).

Table 1 shows the results of our analyses of some types of cell nuclei for per cent DNA and PNA. We do not yet have the corresponding values of DNA per nucleus to complete this table.

In general, the results shown in table 1, when taken together with studies of protein and enzyme extraction from the isolated nuclei by various solutions as shown in table 2, indicate a much greater loss of protein from nuclei prepared in isotonic saline than from nuclei prepared at pH 6 or 4 in distilled water with a small amount of citric acid added. Nuclei isolated in isotonic sucrose at pH 6 are nearly the same as those isolated in distilled water, but nuclei isolated in hypertonic sucrose at pH 6 seem to have lost protein. As we have emphasized previously (Sumner and Myrbäck, '50), nuclei prepared in strong citric acid show the loss of a very great amount of material other than DNA and perhaps should be termed "nuclear remnants" rather than nuclei, in spite of their fine microscopic appearance. Results of studies of PNA and protein using the latter type of nuclei may be extraordinarily misleading. Such nuclei can be used to great advantage, however, in studying certain aspects of DNA and PNA metabolism, although they appear to re-

TABLE 1

DNA and PNA content of cell nuclei prepared in different media
(Parentheses refer to number of determinations)

CONSTITUENT		0.85% SUCROSE	0.88 *M* SUCROSE	DIL. CITRIC ACID, pH 6	DIL. CITRIC ACID, pH 4	DIST. H_2O + $CaCl_2$[a]	0.9% NaCl + $CaCl_2$	BEHRENS
DNA	Schneider	7.9 (7)	11.2 (4)	10.8 (5)	10.8 (5)	13.2 (1)	16.5 (1)	4.4 (2)
	Spectro-photometric	. .	. .	8.5 (6)	10.2 (5)	. .	. .	. .
PNA	Schneider	. .	. .	5.1 (3)	4.0 (1)	. .	. .	. .
	Spectro-photometric	. .	. .	4.5 (6)	7.3 (6)	. .	. .	. .

[a] Catalase and aldolase very low in these nuclei.

TABLE 2

Extraction of nuclei (prepared at pH 6) with different media

EXTRACTING MEDIUM	PER CENT N EXTRACTED	PER CENT P EXTRACTED	PER CENT ALDOLASE EXTRACTED	PER CENT ARGINASE EXTRACTED
pH at 7.0 with dil. NaOH (autolysis)	8	. .	. .	. .
0.9% NaCl (3 ×)	17	27 [a]	60–70	30–40
5.0% NaCl *after* 0.9% NaCl	23	59 [b]	. .	. .
8.5% Sucrose (isotonic)	3	Very low	0	. .
0.88 *M* Sucrose (hypertonic)	4	Very low	0	. .

[a] Contains PNA.
[b] Most of the DNA appears here.
Note: Disintegration of nuclei by sonic vibrations does not alter these results materially.

tain only a firmly bound PNA fraction and not the total PNA originally present in the nucleus, as will be seen shortly.

Studies of the PNA content of cell nuclei prepared at pH 4 indicate a considerably higher percentage than found in nuclei prepared in strong citric acid (Barnum and Huseby, '50). Nuclei prepared with very dilute citric acid at pH 6 have about the same PNA content. Nuclei prepared at pH 6 and subsequently extracted with 0.9% saline lose considerable PNA. It is now known that PNA can be extracted to a considerable extent from whole cells with physiological saline solution (Kerr and Seraidarian, '49). Nuclei prepared by the procedure of Behrens appear to contain even more PNA and DNA (Dounce et al., '50). All this indicates that some PNA is rather easily lost from cell nuclei, and makes it seem possible that the microsome fraction, to be discussed subsequently, might in part be derived from cell nuclei (Schneider and Hogeboom, '51). What has been said also shows that even ultraviolet microspectrography could be unreliable when applied to tissue fixed in strongly acid media.

There has been a widespread notion that absence of PNA in nuclei could be used as a criterion of purity of the nuclei. According to the above suggestions, this idea would be of very doubtful significance. There does, however, seem to be a certain very firmly bound PNA fraction in nuclei which resists extraction with 5% acetic acid and possibly 0.9% saline, and which shows a high metabolic turnover rate as measured by P^{32} uptake (Barnum and Huseby, '50). This fraction might represent nucleolar PNA but this is only speculation.

At least a few enzymes can be extracted by means of physiological saline to the extent of 50% or more from cell nuclei isolated in very dilute citric acid at pH 6. However, a certain portion of a given enzyme usually is quite firmly bound to the nuclear residue and resists extraction.

In regard to soluble enzymes which occur in the nucleus as well as apparently in solution in the cytoplasm, it might be questioned whether an equilibrium exists as the result of diffusion of such enzymes back and forth through the nuclear membrane. No information is yet available on this point.

The histone of the cell nucleus represents an interesting although inhomogeneous protein which is believed to be an exclusive constituent of cell nuclei. This protein is ordinarily firmly bound but can be brought into solution from isolated nuclei by means of 0.2 *N* hydrochloric acid. Recent studies by Miller and Dounce (unpublished) have demonstrated a rapid uptake of lysine containing radioactive carbon by the histone of liver cell nuclei, in resting liver cells, and it might be stated parenthetically that therefore histone should be removed very completely from DNA in turnover studies of the latter material using radioactive carbon or heavy nitrogen.

Work with enzymes, proteins, and nucleic acids of cell nuclei isolated in various ways has convinced us that cell nuclei contain an easily extractable protein fraction which can be brought into solution by extracting with 0.9% sodium chloride or by raising the pH. This fraction may be part of the ''nuclear sap,'' which can be demonstrated in the case of frog egg nuclei, using microdissection techniques (Brown et al., '50). However, it should be noted that the easily extractable protein fraction cannot be made soluble by disintegrating nuclei prepared at pH 6 in distilled water with a sonic oscillator. Hence this fraction must be in a combined state in the latter type of nuclei, possibly combined with nucleic acid because of the pH employed. More or less of the easily extractable protein may be lost from nuclei isolated in aqueous media, depending upon the method of preparation, and probably the only way to recover the entire fraction would be to prepare nuclei by the Behrens technique. Most of the enzymes studied by us seem to be in this easily extractable fraction, although in the case of enzymes like aldolase, arginase, and catalase, it is difficult or impossible to effect a quantitative separation from the nuclei prepared at pH 6 by extraction with saline.

The question remains open as to whether a similar fraction can be extracted from chromosomes. It might be possible to investigate this question by preparing chromosomes in isotonic sucrose solution and subsequently extracting them with saline solution.

It should be of interest to analyze the easily extractable protein fraction of cell nuclei and to compare it with the corresponding fraction obtained from whole cells. Of even greater interest would be to attempt to find the origin of the easily extractable protein fraction. Does it originate in nucleus, in the cytoplasm, or in both locations? It might be possible eventually to throw light on this question by isotope work.

Microsomes. The microsome fraction represents a heterogeneous group of very fine particles rich in PNA and lipid. Until recently they were not thought to possess enzyme activity, but lately several enzymes have been found in considerable concentration in the microsome fraction (Sumner and Myrbäck, '50; Hogeboom and Schneider, '50; Palade, '51). Whether these represent adsorbed enzymes cannot yet be stated with certainty.

APPLICATION OF CELL FRACTIONATION STUDIES TO RADIATION PROBLEMS

Since this symposium deals with radiation it is natural to ask how the material on cell particulates, as outlined, might be applied to studies of the effects of radiation on cells. Since the author knows very little about problems concerned with radiation, only a very brief attempt to show possibilities of such application will be made.

It seems to be generally accepted that sufficiently high dosage of X radiation can affect proteins by bringing about their denaturation, can depolymerize nucleic acids, and can adversely affect a number of other metabolites, often through oxidative changes. The work of Barron and collaborators ('49) indicates that, in medium dosage *in vitro,* X radiation has a marked preferential action on sulfhydryl enzymes, which in the presence of oxygen, seem to be inactivated by oxidation of the -SH groups. It is believed that in this case the primary action of the X rays is to decompose water, giving rise to oxidative free radicals which may do direct damage by chemical action or which may produce damaging secondary products such as hydrogen peroxide. In considering still smaller doses of X radiation, such as might be expected to produce mutation or chromosomal degradation without evidence of gross chemical damage, it seems necessary to consider the possibility of direct hits on affected chromosomes by moving electrons ejected with sufficiently high translational energy from atoms which absorb the radiation.

Returning to damage of -SH enzymes by moderate doses of X radiation, it can be seen at once that the mitochondria should be important sites of X-ray damage in the cell, if the *in vivo* changes correspond to *in vitro* experiments, since the entire amount of succinic dehydrogenase, an enzyme classed with the -SH enzymes, appears to be located in the mitochondria (Schneider and Hogeboom, '50). Destruction of all or most of the succinic dehydrogenase would obliterate one of the most important roles of mitochondria in cell metabolism, namely, that of catalysis in the Krebs carbohydrate oxidation cycle. Whether damage to succinic dehydrogenase could be repaired without the neosynthesis of more mitochondria would probably depend upon such things as the percentage inactivation of the enzyme and the availability of other -SH groups. This point is no doubt susceptible to experimental investigation.

Another enzyme which is even more certainly known to be dependent upon -SH groups for activity is 3-phosphoglyceraldehyde dehydrogenase, a member of the glycolysis cycle. Although, as might be predicted, the bulk of this enzyme probably occurs in cytoplasm, presumably in the solution phase, it can also be easily demonstrated in liver cell nuclei (Dounce, '50) and if its presence is essential there, it follows that destruction of the enzyme by X radiation could constitute one possible mechanism for damage to cell nuclei, as well as to cytoplasm.

Proteolytic enzymes (some of which may depend upon -SH groups for activity) are to be found in the nuclei of liver cells as well as the cytoplasm (Miller and Dounce, unpublished research). Destruction of activity of a proteolytic enzyme by oxidation of -SH groups might be responsible for damage to both cytoplasm and nuclei.

These three examples of enzymes susceptible to X irradiation indicate that radiation might be able to exert action on almost any part of the cell, as far as damage by the chemical route of -SH oxidation is concerned. There does not seem to be any reason a priori to think that one part of the cell should be particularly more sensitive than another. However, severe damage to cytoplasm and to mitochondria in particular, might be expected to destroy the cell very quickly.

A recent paper dealing with nonsulfhydryl enzymes shows that the intracellular distribution of catalase, esterase, arginase, rhodanese, and alkaline phosphatase in rat liver was not affected by total body irradiation with 500 r, nor were the total concentrations of these enzymes affected (Ludewig and Chanutin, '50). It would appear to be of somewhat greater interest, however, to study the intracellular distribution of -SH enzymes after irradiation.

The use of cytochemical procedures also seems warranted in attempting to elucidate the gross effects of radiation on nucleic acids which have been reported in the literature (Lea, '46).

When small doses of radiation are in question, such as can bring about detectable chromosomal or gene damage without immediate gross effects on the cell, it seems likely that serious direct damage to cytoplasm should be negligible, owing to the repetition of vital enzyme structure throughout the cytoplasm. Damage to an enzyme in a limited number of mitochondria, or even outright destruction of some mitochondria, could hardly be expected to cause irreversible cell damage. This idea is supported by work with denucleated half eggs of the sea urchins, which do not suffer a delay in cleavage following fertilization as the result of X irradiation, although a delay is observed after irradiating sea urchin eggs containing the nucleus (Lea, '46). Hence it appears to the writer as though study of the mechanism of damage to cells by radiation perhaps should be divided at the outset into two categories: (a) cases where the dosage is high enough to bring about measurable damage to -SH enzymes or other metabolites anywhere they are found in the cell, with resultant primary damage to cytoplasm as well as nucleus; and (b) cases where only minimal or negligible primary damage to cytoplasm is to be expected but where primary damage to the nucleus is brought about, presumably through a chromosomal or gene mechanism.

If primary damage to a cell is restricted to damage of a chromosome or destruction of a gene, the interesting question arises as to how and when such damage may bring about more apparent and general cellular damage. Some work (Sumner and Myrbäck, '50) has been reviewed indicating that genes may exert their biochemical actions during the resting state of the cell, rather than during the dividing state. From the work of Beadle and collaborators (cited in Sumner and Myrbäck, '50), this becomes easily comprehensible on the basis of a one-to-one correspondence between genes and enzymes. However, even if this correspondence is interpreted as meaning the synthesis of a given enzyme by the action of a corresponding gene (presumably with the aid of a more general synthetic mechanism also present in the cell), it does not follow that destruction of a gene would bring about the immediate loss of a corresponding cellular enzyme, for several reasons. In the first place, genes occur at least in duplicate in the diploid or polyploid somatic cells of animals, so that destruction of one of a corresponding pair of genes might only produce something amounting to a recessive character which would be difficult or impossible to detect without genetic studies.

In the second place, even assuming a destruction of both genes corresponding to a given character, the effect on cellular metabolism might be delayed because of the possible intermediary action of "plasma genes" (references listed in Sumner and Myrbäck, '50).

Finally, even assuming the obligatory direct participation of the gene pair in question in synthesis of a given enzyme, and also assuming destruction of both members of the pair, it might take some time to exhaust the reserve of the enzyme in question to the point where an effect upon cellular metabolism would be evident. Experiments with denucleated amoebas (reviewed in Sumner and Myrbäck, '50) favor this last explanation.

Arguments of the sort just proposed indicate the necessity for gaining further knowledge of the biochemical mechanisms whereby cell nuclei exert influence over cellular metabolism, if we are to fully understand the mechanism of damage to the cell induced by small doses of radiation. The problem is so difficult that all possible routes of approach should be used. No single approach is likely to furnish sufficiently decisive or complete information, and arbitrary rules of procedure, such as deciding to ignore the presence of enzymes in cell particulates when these particulates do not happen to contain the major portion of the cellular enzymes in question (Schneider and Hogeboom, '51), seem to the author more likely to retard rather than to advance research in the field.

DISCUSSION

CHAIRMAN CARTER: The paper is now open for discussion.

TIPTON: I realize that most of this work is done with large groups of nuclei. Do you think that any individual work with individual nuclei is possible? I feel sure that at different stages in mitosis etc., the condition of the nucleus must be quite different. Might it not be possible, on a small scale, to investigate individual nuclei? I also wonder if there are any energetic reactions in the nucleus at all.

You say succinic dehydrogenase and some of the flavoproteins are in mitochondria; yet certainly the nucleus is a very active place at times. Have you any suggestions of what enzymes one might look for in the nucleus?

DOUNCE: In regard to single nuclei, it is certainly possible to do some work; in fact, some has been published. Recently I read a paper on the composition of nuclear sap of the large nuclei of frogs' eggs, obtained, of course, by microdissection. The nuclear sap is found to contain protein, but I do not think any enzyme analyses were done. As a matter of fact, I believe the soluble saline fraction obtained from liver nuclei may represent the protein of the nuclear sap. Brachet and Holter and Linderstrøm-Lang have done work with nuclei of single-celled organisms, obtained by microdissection. Therefore work can be done with a single nucleus but whether it is possible with single mitotic nuclei, I am not so sure.

In regard to enzymatic reactions furnishing energy, we have found quite a number of glycolytic enzymes in nuclei. Of course there is a dispute as to whether the presence of these enzymes in isolated nuclei means anything. I am convinced these enzymes do not represent contamination; concentrations found are too high for that. But then the questions arises as to how they got there. Did they diffuse into the nucleus from cytoplasm either in the intact cell or during preparation of the nuclei, or were they generated in the nucleus to be thrown out into the cytoplasm subsequently? In a search for enzymes which can furnish energy, I think you must investigate the glycolytic enzymes; the Krebs cycle is not present in the cell nucleus, as far as I can see. We found cytochrome oxidase in a measurable amount in isolated nuclei and do not think that this is due to contamination, since no succinic dehydrogenase was found in the same nuclei. But what function the cytochrome oxidase could have in the nucleus I cannot imagine.

TIPTON: Are any of the cytochromes in the nucleus?

DOUNCE: There is a small amount of cytochrome c. I did not measure it quantitatively, since I do not consider existing methods very accurate for measuring cytochrome in very small amounts.

Carter: Have you found cytochrome c in the nucleus, Dr. Tipton?

Tipton: No, we have not tried it.

Dounce: Did you try the glycolytic enzymes?

Tipton: No, that was the reason I was asking.

Sheppard: An interesting question arises, when you consider the pliability of cellular materials, as to what would be the effect of doing some of these separation procedures at low temperatures. This, of course, brings up several technical problems, one being the maintenance of low temperature during the homogenization process; any other being the sudden lowering of the temperature, or otherwise providing some material in the medium that will preserve the vitreous state and not allow ice crystals to form. If you broke up tissues at a low temperature, do you suppose that the fracturing mechanism would proceed in such a way that the cell constituents would separate cleanly? Or would the fractures tend to go diagonally across the structural features and disrupt individual structures?

I mention the question of low temperature since I am not sure that all biologists realize how easy it is to do work at lower temperatures. Liquid air as well as liquid nitrogen is now becoming quite cheap.

Dounce: I can hardly answer the question. Working with Behrens' type nuclei, we ground the tissue at — 15°C. and subsequently fractionated them at 0°. But the nuclei remained more or less intact, although some were broken. The broken fragments are troublesome, because, in Behrens' technique specific gravity flotation as well as differential sedimentation is used, and the broken pieces tend to accumulate with the nuclei, making it difficult to decide for certain whether cytoplasm is also present.

At any rate, you can grind lyophilized tissue in a ball mill at — 15°C. and obtain whole nuclei. Whether you can do the same at lower temperature, I do not know.

SHEPPARD: I am thinking of chopping up the tissue in liquid air without previous lyophilization.

DOUNCE: It is not exactly a question of chopping up tissue; you must really pulverize it to break off the cytoplasm. I did not mention that, in some tumor cells, cytoplasm is very resistant. However, I do not know of any reason why Dr. Sheppard's proposal could not be accomplished. I see no obstacles except technical ones which probably could be overcome.

FURTH: It would be helpful for us morphologists if we could have some idea about the ultramicroscopic picture of not only the nuclei but also of other particles, including the nucleolus, and whether the latter is a morphological entity.

DOUNCE: Cytologists would probably answer this question much better than I. The microsomes probably comprise a range of particles that are just at and below the limit of resolution in the light microscope, so that a dark field illuminator is needed for observing them. The microsomes contain a high percentage of pentose nucleic acid — 10–15% or more, and therefore should be, I suppose, basophilic. An attempt to identify microsomes in sections may show agglutination. I would think that the electron microscope is about the only instrument capable of throwing light in the ultrastructure of microsomes. As for the nuclei, once they are isolated — depending upon the method of isolation — they can be quite rubbery and gelatinous and very difficult to disperse. Isolated in such a way as to inhibit enzymatic action, at pH 4 with citric acid, or by means of the Behrens technique, the nuclei are quite gelatinous. It seems very difficult to separate the constituents of such nuclei. But with nuclei at pH 6 which are not gelatinous, the individual chromosomes can be shaken out by means of sonic vibration.

I believe that the nucleolus is an individual unit, because I can see individual nucleoli after breaking up the nuclei in an oscillator. I know nothing of the ultrastructure of nucleoli. The ultrastructure of chromosomes is well known to cytologists. The ultrastructure of mitochondria is unknown to me.

CARTER: Is the PNA of the nucleus confined exclusively to the nucleolus?

DOUNCE: I should doubt that there could be enough nucleolar material in nuclei to account for it. According to Barnum and Huseby, the PNA fraction obtained by repeatedly washing the nuclei in about 3% citric acid, which I think takes out some PNA, is very active metabolically. Possibly this fraction represents nucleolar PNA, but this is merely speculation. The chromosomes, of course, contain some PNA, in spite of having been washed with isotonic saline. I doubt very much that all the PNA is in the nucleolus.

CARTER: Doctor Vloedman, did you have something to say about this turnover of PNA in the nucleolus?

VLOEDMAN: The only information that I have on this point is from Barnum's work using P^{32}, in which he found a very high turnover rate from the PNA in the nuclei. However, Raghardt has done very much the same sort of thing using C^{14}, and he could find no essential difference in the turnover rates of PNA in various cell components.

HOLLAENDER: The nucleolus is in itself by far the most sensitive structure to ultraviolet radiation. There must be some unstable components in the nucleolus, at least with respect to ultraviolet radiation, or else the ultraviolet must be more highly absorbed by the nucleolus. Photoreactivation of the nucleolus can be observed under the microscope.

HASSET: I can tell you now that if you are going to work with liver tissue or something similar, you probably will not be able to use the device for breaking cells described in our paper. Higher pressures are necessary.

The method has been tried on liver tissue at Jefferson Medical College and it does not give 100% cell disruption. We used a maximum pressure of 2000 pounds, but by using a pump and strong cylinders, higher pressure could be obtained. Oddly enough, the tissue is not blown out of the apparatus on decompression; where originally there was a mass of tissue, a soup remains.

Ferguson: It appears that, owing to intracellular organization, it is possible for antagonistic reactions to proceed at the same time within the cell. But in extracts of whole cells, there may be inhibitors of an enzyme together with the enzyme, and some of our most active preparations of activators for blood-clotting systems also contain quite appreciable amounts of inhibitors. I think that this phenomenon raises a fundamental point concerned with the breaking down of the cell and subsequently isolating the components. One biochemical approach to the problem may be to obtain separation of antagonistic materials as the result of cytochemical separation of the cell particulates.

Dounce: Unfortunately, I do not know anything about this problem, but the question raised is very interesting.

Sparrow: I would like to ask Dr. Dounce what is the yield of isolated chromosomes compared to the total amount of nuclei or DNA? Does it agree roughly with the number of cells in division?

Dounce: I have not measured the yield, but there are few if any nuclei in our preparations that come from dividing cells. We have simply observed that the nuclei are broken by the sonic oscillator and that some chromosomes and nucleoli are liberated. There is apparently no dry weight at all in the supernatant after spinning at approximately 18,000 r.p.m., as long as no salt is added.

The suspension that comes out of the oscillator consists of broken pieces, whole chromosomes, nucleoli, and extremely fine suspended material.

Sparrow: I would also like to point out, in case anybody is interested, that it is quite easy with a number of plants to isolate pollen mother cells in 10–100 mg quantities. Individual stages of mitosis can be studied. I have never worked systematically on isolation of chromosomes or nuclei from such material, but in the routine handling of the material, cells often break open and the chromosomes pop out and are lost. This phenomenon might be of use to biochemists.

KRAKAUR: While I was working with Dr. Green at Columbia, we developed a method for separation of cell particles which has certain drawbacks which we have not been able to iron out. It is incomplete and we have not published it for that reason. But it has certain applications to some of the questions that were brought up today.

Doctor Dounce mentioned that a solution of 30% sucrose is sticky. We used even higher concentrations of sucrose, up to 70%. Such solutions are extremely sticky, yet we were able to get excellent separations of all cell components at 14,000 g in a type of angle centrifuge. Minimal loss of physiological activity occurred, as was indicated by studies on the cyclophorase system.

The procedure was one not of differential centrifugation but of utilizing differences in specific gravities. One of the drawbacks of the procedure is that sucrose may dehydrate the particles, and that is something we have not completely investigated. We were using rabbit liver. We identified a particle in the mitochondrial preparation which causes the tannish color of mitochondrial preparations as a separate fraction, and it resembles material described by one author as Golgi bodies, that is, the round precursors which are changed by osmic acid to a coalesced material. The preparation of nucleoli that we obtained by this procedure was about 80% pure. The problem here is one of fractionation and working rapidly at low temperatures. Concerning the physiological activity of particulates, with 50% solution of salt-free sucrose the entire homogenate, when washed free of sucrose, had about 80–90% of the total cyclophorase activity of the control preparation. However the homogenate had no activity when amounts of sucrose were greater than 2% in the preparation analyzed.

Therefore, I believe that the problem is to be sure of washing out the sucrose completely.

Krakaur: Although there is this problem of stickiness when working with high concentrations of sucrose, the morphology of the nuclei does not seem to be changed completely. There are two further advantages which I should mention. All the whole cells come to the surface with the first spinning when 70% sucrose solution is used at the start of the experiment. Clean nuclei sediment and those with any cytoplasm attached remain in the supernatant.

Although our yields are very low and we have, in a whole homogenate, very few nuclei with the usual Waring Blendor procedure, if you use a large enough amount of material, you probably can obtain a large quantity of nuclei without any signs of tabs. Apparently not very much protein is extracted by this procedure. Therefore, I am wondering whether anyone might be interested in trying the method. As I say, it has not been published.

The problem was such a tremendous one that we were content just to demonstrate that we could get approximately 80% nucleoli. We never went further with any of the fractions; we did not attempt to identify any metabolic activity with the nucleoli.

Ashwell: I would like to take up the question of radiation in regard to mitochondria. The only work in this connection that I know about was done by the University of Chicago group, some of whom are here. As I recall, they radiated whole animals, and in homogenate studies on the tissues could find no effect on the succinic dehydrogenase. In our laboratory we have commenced work on isolated mitochondria which have been subjected to radiation in order to determine whether or not there is any effect on the succinic dehydrogenase system.

As yet our results are incomplete, and we are not quite certain where we stand. I wondered if anybody else in the audience is working on this problem, and whether they have any further results to report?

Carter: Doctor Patt, could you make a few remarks about Dubois' work?

Patt: Doctor Coon is here and I think perhaps he ought to comment on Dubois' work. I would like to say that Dr. Gromet, who is with us at the Argonne Laboratory, made some studies on the succinic dehydrogenase system in rat kidney and failed to observe any inactivation of this enzyme system. Doctor Coon, I think this essentially confirms the negative results of Dubois.

Coon: I can only say that we do not think that there is a discrepancy between Dubois' and Barron's work because of the completely different conditions under which the two pieces of experimental work were carried out. Doctor Barron, as I understand, was working with very large doses of radiation on the purified enzyme system. Is that correct?

Patt: No; he also determined the effects on tissues from irradiated animals, in addition to his work with purified enzymes.

Coon: But with much larger doses, I believe.

Carter: Up to 500 r.

Coon: Then you would call this a discrepancy?

Patt: I would.

Coon: Then I have no way to explain the differences in results. It is unfortunate that Dr. Dubois is not here himself, since I think he could contribute to this point.

Patt: I might call attention to a paper in the Proceedings of the Society for Experimental Biology and Medicine, by Dunning, and several collaborators, I believe, which appeared several months ago. These people, interested primarily in bone marrow, reported a decrease in the activities of several enzymes in the bone marrow of chickens. I do not know how one can account for these discrepancies other than to say that the selective inhibition of sulfhydryl enzymes is certainly open to some question.

Dounce: It looks as though if mitochondria are isolated and irradiated *in vitro*, a relatively small inhibition of certain -SH enzymes does occur — of an order of magnitude of 15–25%, and quite variable. Of course, the techniques are quite different depending upon whether purified enzymes or mitochondria are irradiated, or homogenates of tissues of irradiated animals are studied. Barron, I believe, studied tissue homogenates rather than isolating the mitochondria.

Carter: The revolving platinum electrode can be used to titrate -SH groups. I am wondering whether anyone has conducted experiments with this electrode to see whether, subsequent to irradiation, there is a change in titratable sulfhydryl groups. Does anyone have a comment to make?

Boell: I will speak of this in my talk this afternoon, but I think part of the discrepancy in Barron's results on purified enzymes and results of studies on whole tissues can be resolved by the fact that, under certain circumstances, the enzyme being studied is set up to be the limiting factor in the reaction measured. However, in tissues it is unlikely under many circumstances that the enzyme in question is present in sufficiently dilute concentrations to be a rate-limiting factor. Therefore, in a whole tissue, one may have a considerable amount of inactivation of a given enzyme without there being any demonstrable effect upon the total metabolism of that tissue.

Patt: To attempt to answer your question of the changes in sulfhydryl content of tissues, a study was made at Hunter's Point, San Francisco, although I do not recall the name of the investigator. Sulfhydryl groups in plasma of irradiated animals were titrated and little change was found for the first day or two; then a decrease in the sulfhydryl content occurred in the intermediate and late periods after radiation, which I would interpret as a fairly nonspecific change, since it has been observed in a variety of conditions.

We have used a somewhat less elegant procedure to titrate sulfhydryl groups in a number of tissues, including thymus, small intestine, kidney, and bone marrow, and we have been interested only in immediate changes in sulfhydryl levels. We failed to observe any immediate changes, either decreases or increases, in amount of sulfhydryl groups.

Actually, of course, this is not inconsistent with theoretical considerations because, the computed expected oxidation of sulfhydryl groups would be an exceedingly small fraction of the total available sulfhydryl reservoir. Therefore, I do not know what such studies prove.

DOUNCE: Can you titrate protein-SH groups with the rotating platinum electrode?

CARTER: Proteins have been studied in this way by a group at Johns Hopkins, and now a group at Boston is studying components of the red cell.

DOUNCE: How do you account for the fact that this can be done? What is the electrochemical change? Are S-S bonds being formed, and if so, does that mean the linking of two protein molecules, or are two groups in the same protein molecule reacting? What is the mechanism?

CARTER: I would not be in a position to say. Another of your comments that might deserve some discussion, is that the DNA is a metabolically inactive compound in the resting cell. This depends on the radioactive precursor you use. With phosphorus, Barnum (published work) gets high PNA and low DNA turnover. But using glycine or formate as a precursor, DNA turns over just as rapidly as the pentosenucleic acid. This is true in the resting cell.

STREHLER: With respect to the assay of succinic dehydrogenase activity and other enzymes, we in the Biochemistry Section here have been concerned, among other things, with the utilization of firefly luminescence, as a tool for studying energy-transfer pathways.

In some preliminary studies, which were intended largely to demonstrate the feasibility of the method, it was shown that mitochondrial preparations prepared by the method of Green, would oxidize various intermediates of the Krebs cycle, with concomitant formation of ATP, which could then be measured with a photomultiplier using the firefly luminescent system as the assay tool.

In brief, the results were that succinic acid seemed to be the best substrate in terms of rate of formation of ATP. P/O ratios calculated from the data obtained were phenomenally low; and inasmuch as this is a mixed system in which the firefly enzyme is mixed with the mitochondrial preparation, one cannot easily compensate for such hydrolysis for high energy phosphate esters as may occur. It is therefore difficult to interpret these P/O ratios.

However the question of further transformations of the energy-yielding hydrogens in oxidizable substrates, that is, what happens to their energy subsequent to the removal of hydrogen from the substrate, seems to me to be a subject possibly relating to radiation damage. By examining oxygen consumption alone, one certainly has a very incomplete picture of the relationship of succinic dehydrogenase or any -SH enzymes to the general metabolism of the organism.

Dounce: I would like to ask Chairman Carter one more question about the formation of DNA from various radioactive precursors. Do you conclude that the DNA is not totally broken down and synthesized in resting cells but that only pieces of it are being broken down and rebuilt?

Carter: Yes; and I think that conclusion is reasonable in view of some recent experiments reported by Buchanan. Therefore we must look upon DNA perhaps as having a pattern just like a protein; but within that pattern there is metabolic lability.

Levedahl: Doctor Dounce, would you comment on your method of preparation of the sample for the catalase assay?

Dounce: We simply suspended the whole nuclei in hydrogen peroxide.

Levedahl: What was the temperature used? Were the nuclei in 0.9% sodium chloride?

Dounce: We have a method for determining catalase at room temperature, gasometrically, and also have determined it at 0°C. The nuclei were in distilled water, but I do not believe that the use of 0.9% sodium chloride would change the results.

Levedahl: At UCLA we have done a little work with catalase activity, and we have been in the rather embarrassing position of finding about twice as much activity as everyone else reports, by modifying our preparations in certain ways. Also another point that should be brought up is that, in preliminary work, we have found that the nutritional state of the organ can influence catalase level by perhaps 300 or 400%. This is in kidney. In liver the effect is much lower. I meant to ask where the nuclei came from.

Dounce: They came from liver. We have found that catalase and aldolase activities are lower in liver nuclei prepared in sucrose solutions.

We used these two enzymes in order to find out the results of preparing nuclei at a pH above 6. These enzymes are low in concentration in nuclei prepared at high pH. I think the catalase was only one-third to one-half as concentrated as in nuclei prepared at pH 6. The aldolase was even lower in concentration, perhaps a fifth to a quarter as concentrated. That point is rather interesting, and may mean one of a number of things. It could mean that at high pH enzymes present in the nuclear sap are extracted from the nuclei and lost. Or it may be that a lower pH caused more binding of catalase by nucleic acid of the nuclei. There are various possible interpretations. We have done considerable work during the war in analyzing for catalase, and have worked out a gasometric method which gives roughly quantitative results at room temperature. You can use a temperature of exactly 25°C. if you want slightly better precision. But the variations in tissues are great enough as far as we are concerned not to warrant using a titrametric method at 0°C.

Levedahl: Our method is gasometric also.

Dounce: You have to be very careful not to make the substrate too concentrated. You can get into trouble with the gasometric methods if you do not calibrate them pretty carefully. That is, the activity may not be proportional to the amount of enzyme, and so on.

I might say that in working with these nuclei we have nearly always used the whole nuclei for enzyme tests; very seldom have we tried to grind them up. With one enzyme (phosphorylase) a greater activity was obtained after grind-ing, but in nearly all cases we use the suspension of whole nuclei and assumed that the substrate would rapidly diffuse to the enzyme in case the enzyme did not all dissolve in the medium. Perhaps that assumption is not justified, however.

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ORGANIZED ENZYME SYSTEMS

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The concept of organization is a basic one in the biological sciences. The student of biology soon becomes aware that the organism is more than the sum of the individual organs and the organ more than the sum of the component tissues. The arrangement and integration of parts at each level of complexity constitutes that which the biologists call organization. How far down in the biological scale does the concept of organization extend? There has been and still is a school of thought which maintains that the organizational component disappears at the level of the individual enzyme. According to this view, each enzyme is a complete unit in the sense that all the enzymatic events of the cell can be reconstructed by suitable combinations of individual or separate enzymes. Whatever the geography or the arrangement of enzymes in the cell, there is no process or sequence which cannot be duplicated by summation of the component enzymatic activities. This bold approach to enzyme chemistry has paid off excellent dividends. Since the early 1930's the cell has been assaulted by a variety of means, and literally hundreds of different enzymes have been extracted, separated, crystallized, and characterized. The entire process of glycolysis has been reconstructed by adding together some 20 individual enzymes, each of which was prepared in a highly purified form. The synthesis of starch, glycogen, disaccharides, and dextrins can now be accomplished in relatively simple systems of enzymes. Is there any reason therefore to invoke the organizational component at the level of the enzyme system? In recent years the study of the enzyme systems involved in the citric acid cycle has uncovered some facets which bear on this problem of organization. Many laboratories have contributed to the results which I propose to bring to your attention, though limited time will not permit me to do full justice to all the pioneer investigations in this field.

In 1946 we had occasion to study the oxidation of pyruvate in homogenates of rabbit kidney. All efforts to isolate an oxidase which catalyzed the one-step oxidation of pyruvate to acetate proved unsuccessful. Regardless of the fractionation procedure employed, fractions were either inactive toward pyruvate or, if active, contained all the catalysts necessary for the complete oxidation of pyruvate to carbon dioxide and water. It should be pointed out that the activity of the pyruvic oxidase system could be preserved only by working within rather narrow limits of temperature, hydrogen ion, and salt concentrations. This relative instability of the system admittedly limited the scope of the permissible fractionation procedures. The oxidase system was found to be associated with relatively gross granules or particles which were sedimentable at low centrifugal speeds.

As a result of our studies on the properties and general behavior of the pyruvic oxidase system, we were led to the conclusion that the system was not a random collection of separate, discrete enzymes, but was an organized complex of enzymes. The proportions of the different enzymes of the complex were presumed to be fixed and, within limits, not capable of alteration by the experimental conditions to which the complex was exposed. In other words, the enzyme unit was no longer the individual enzyme, but a hierarchy of enzymes bound together into some structural mosaic. Since the oxidation of pyruvate proceeded by way of the citric acid cycle, the complex of enzymes was named in 1947 ''the cyclophorase system''[1] (Green et al., '47; Green, Loomis, and Auerbach, '48).

[1] At the time the concept of the cyclophorase system was introduced there were several investigators, notably P. P. Cohen, F. E. Hunter, A. L. Lehninger, L. Leloir, R. A. Peters, and V. R. Potter, who had already carried out extensive studies of metabolic processes in particulate systems, and while they did not specifically postulate an integrated complex of enzymes, it would appear that such a concept was a tacit working hypothesis in their experiments. These investigators have laid the basis for most of our present knowledge of the metabolic processes in particulate systems.

The concept of the organized enzyme complex was not a familiar one in the field of enzymology, apart from scattered observations the import of which was not fully appreciated, and it must be imagined that we had to consider our reasons for proposing so unorthodox an interpretation as very compelling ones. The basis for the cyclophorase concept rested upon the following considerations which have to do exclusively with the behavior of the enzyme complex.

Complete repertoire of enzymes. All the enzymes necessary for implementation of the citric acid cycle are found together in the same particulate fraction (Green, Loomis, and Auerbach, '48).

"Perfect" proportions of enzymes. The relative proportions of the various enzymes are so nicely adjusted that any member of the citric acid cycle can be oxidized to carbon dioxide and water without any significant accumulation of intermediary products (ibid.).

Full complement of enzymes. The enzymes of the cyclophorase complex contain all the coenzymes necessary for attainment of maximum activity. With the exception of inorganic phosphate, adenosine-5-phosphate, and magnesium ions, no requirement is demonstrable for any of the group of coenzymes known to participate in the reactions of the citric acid cycle. The necessary coenzymes are intimately bound to their appropriate apoenzymes within the cyclophorase complex (ibid.; Still, Buell, and Green, '50b).

Interdependence of reactions. Many of the processes catalyzed by the cyclophorase complex of enzymes, e.g., fatty acid oxidation, have to be initiated by the cooxidation of members of the citric acid cycle (Grafflin and Green, '48; Knox et al., '48). The analysis of this "sparking" phenomenon leads to the conclusion that one set of reactions brings about the "activation" of a coenzyme, and in another set of reactions the activated coenzyme reacts with the substrate. Since no addition of external coenzymes is necessary for the sparking phenomenon to take place, a reasonable conclusion is that the complex of enzymes enjoys a type of structure or chemical organization which permits the sharing of certain coenzymes or components by groups of enzymes within the complex.

Special properties of enzymes within the complex. Many of the component enzymes within the cyclophorase complex exhibit properties which they no longer possess when separated from the complex and isolated as soluble enzymes. For example, the citric, malic, and α-ketoglutaric oxidase systems can esterify inorganic phosphate coincident with the oxidation process when present in the cyclophorase complex, but lose this capacity when prepared as discrete, soluble enzymes (Cross et al., '49). The special properties which enzymes possess as constituents of the cyclophorase complex argues some organization which is not to be found in the isolated enzymes.

The above enumerated considerations led us to deduce that an enzymatic unit existed which encompassed all the enzymes concerned in the implementation of the citric acid cycle and in processes which depend upon the operation of the citric cycle or whose reaction products feed into the cycle. The nature of the structural unit which corresponded to the enzymatic unit was not known at the time the cyclophorase concept was proposed. However, it was the concept of the enzymatic unit which led to the postulation of a structural unit, and not vice versa. The concept of the enzymatic unit rested entirely upon considerations of enzyme dynamics. The particulate preparations from rabbit kidney and liver which we used for studying the cyclophorase complex of enzymes were clearly inhomogenous with respect to their particle types. But since no part of the argument rested upon the homogeneity of the particulate suspension with which the cyclophorase complex was associated, or for that matter, upon identification of the structural unit which corresponds to the cyclophorase complex, the task of purifying the cyclophorase complex was postponed until more information was available on its basic properties.

There appears to be an unfortunate misconception in some quarters that the cyclophorase system refers to a particular way of preparing the complex of enzymes involved in the implementation of the citric acid cycle. The term "cyclophorase" refers to a complex of enzymes, and not to a method of preparation. There are all types of cyclophorase preparations ranging from crude to essentially homogenous, just as any other type of enzyme system can be prepared at all levels of purity.

METABOLIC SPECTRUM

Investigations since 1946 have uncovered many reactions catalyzed by the cyclophorase complex other than those directly concerned with the citric acid cycle. Consequently, the term "cyclophorase system" has come to mean the complex of enzymes which catalyzes not only the reactions of the citric acid cycle, but a variety of other processes which depend directly or indirectly upon the operation of the citric acid cycle. The known reactions catalyzed by the cyclophorase system may be summarized as follows.

The citric acid cycle. One of the first processes to be studied in detail was the oxidation of pyruvate to carbon dioxide and water. Considerable evidence was collected to show that the pathway for the oxidation of pyruvate in the cyclophorase system was identical with that of the Krebs citric acid cycle. Every postulated intermediate of this cycle was burned to carbon dioxide and water, and there was considerable uniformity in the rates of oxidation of all the intermediates. By the use of inhibitors or by variation in the experimental condition, each of the one-step reactions postulated by the Krebs citric acid cycle could be demonstrated in the cyclophorase system (Green et al., '48). Thus the cyclophorase system was identified as the system responsible for the implementation of the citric acid cycle in precisely the fashion deduced by Krebs from his study on tissue minces. It should be pointed out that other workers in the field who have cencerned themselves with the properties of mitochondria have relied to some extent upon the evidence stemming from studies on the cyclophorase system, in assuming that the oxidation of pyruvate in particulate systems does indeed proceed by way of the Krebs citric acid cycle.

Oxidation of amino acids. Kidney and liver cyclophorase systems catalyze the complete oxidation of L-alanine (Still et al., '50a), L-aspartate (Nakada and Weinhouse, '50), L-glutamate (Still et al., '50b), L-proline (Taggart and Krakaur, '49), DL-serine (Mahler and Osborne, unpublished observations), and D-aspartate (Still et al., '49), to carbon dioxide and water. The principle is the same in each case. The oxidation of these amino acids leads to the formation of some member of the citric acid cycle which in turn is burned to completion. Thus L-proline is oxidized to α-ketoglutarate by way of pyrroline carboxylic acid and glutamate; D-aspartate is oxidized to oxalacetate; DL-serine to pyruvate; L-alanine and L-aspartate are converted to pyruvate and oxalacetate respectively by transamination with α-ketoglutarate. In this instance, catalytic amounts of α-ketoglutarate are necessary to initiate the oxidation process.

Hird and Rowsell ('50) have recently discovered in cyclophorase preparations an enzyme which catalyzes the transamination between phenylalanine or tyrosine on the one hand, and α-ketoglutarate on the other with the formation of phenylpyruvate or *p*-hydroxyphenylpyruvate and glutamate. There are no data concerning the subsequent metabolism of these phenyl keto acids in the cyclophorase system.

Fatty acid oxidation. The oxidation of fatty acids in particulate preparations from animal tissues was discovered by Leloir et al. ('39), and then studied in detail by Lehninger et al. ('46). It was early recognized in our laboratory that the cyclophorase complex embraces the group of enzymes which collectively implement fatty acid oxidation (Grafflin and Green, '48). The sparking phenomenon referred to previously provided the clearest evidence of the intimate association between fatty acid oxidation and the cyclophorase system.

The space limitations of the present review preclude any systematic discussion of fatty acid oxidation. However, the salient features of fatty acid oxidation pertinent to the purposes of this review can be summarized briefly. There are three main component processes underlying fatty acid oxidation: (1) initiation or sparking of fatty acid oxidation by cooxidation of some member of the citric acid cycle, (2) β oxidation of the fatty acid to the β-keto acid, and (3) transacetylation of the β-keto acid with oxalacetate to form citrate and a fatty acid with two carbon atoms less than the parent acid. The oxidation of members of the citric acid cycle is accompanied by the formation of some high-energy phosphate ester from inorganic phosphate. The initiation of fatty acid oxidation appears to be dependent upon the formation of a phosphorylated form of some coenzyme and probably involves the interaction of the fatty acid with the phosphorylated coenzyme. The product of the reaction between fatty acid and phosphorylated coenzyme constitutes the activated fatty acid which is the proper substrate for the fatty acid oxidase. The activated fatty acid then undergoes β oxidation to the β-keto acid presumably by way of the α-β unsaturated acid. The entry of the β-keto acid into the citric acid cycle comes about by the following transacetylation reaction:

$$\mathrm{CH_3CH_2CH_2COCH_2COOH + COOHCOCH_2COOH \rightarrow CH_3CH_2CH_2COOH}$$
$$\mathrm{+\ COOHCOH(CH_2COOH)CH_2COOH.}$$

Thus in two of the three phases of fatty acid oxidation an intricate collaboration between a considerable number of enzymes is required. It is little wonder that considerable difficulty has attended efforts to obtain a soluble fatty acid oxidase from animal tissues. Our present picture of the mechanism of fatty acid oxidation calls for an organized complex of enzymes with both enzymes and coenzymes in an orderly arrangement and sequence.

The complete oxidation of propionate in the liver cyclophorase system involves an analogous sequence of reactions (Huennekens et al., '51a, b). There are at least 4 component processes: (1) initiation of propionic acid oxidation by cooxidation of some member of the citric acid cycle; (2) oxidation of propionic acid to L-lactic acid via acrylic acid; (3) racemization of L-lactic acid; and (4) oxidation of D-lactic acid to pyruvic acid, and eventually to carbon dioxide and water via the citric acid cycle.

Synthetic reactions. The cyclophorase system of rabbit kidney and liver catalyzes a series of synthetic reactions which have the following general characteristics. They do not proceed unless sparked by the cooxidation of some member of the citric acid cycle and they are all inhibited by dinitrophenol, a reagent which interferes with oxidative phosphorylation (Grafflin and Green, '48; Cross et al., '49). The known synthetic reactions are the following:

1. acetate + oxalacetate → citrate (Grafflin and Green, '48; Elliott and Kalnitsky, '50).
2. acetoacetate + 2 oxalacetate → 2 citrate (Grafflin and Green, '48).
3. benzoate + glycine → hippurate (Cohen and McGilvery, '46; Kielley and Schneider, '50; Sarkar et al., '51).
4. ornithine + NH_3 + CO_2 → citrulline (Cohen and Hayano, '47; Cohen and Grisolia, '50; Leuthardt and Müller, '48).
5. benzoate + ornithine → ornithurate (McGilvery and Cohen, '50).

Metabolism of C_1 compounds. Recent studies in our laboratory have disclosed that the cyclophorase complex of kidney and liver contains a group of enzymes which are involved in the oxidative phase of the metabolism of C_1 compounds. These enzymes catalyze the following reactions: (1) the conversion of serine to glycine and formate (Sarkar et al., '52); (2) the oxidation of sarcosine to glycine and formaldehyde (Sarkar et al., '52); (3) the condensation of formaldehyde with pyruvate (Hift, '52).

BOUND COENZYMES

The reactions of the citric acid cycle and all other processes catalyzed by the cyclophorase complex of enzymes proceed maximally in complete absence of added coenzymes. Furthermore, added coenzymes in no way affect the course of these reactions other than by inhibition. It would be anticipated therefore that analysis of the particulate suspension which contains the enzyme complex would reveal substantial amounts of all the necessary coenzymes and cofactors. These analyses have been carried out in our laboratory in a series of investigations (Teply, '51). Rabbit kidney cyclophorase gels have been found to contain considerable amounts of pyridine nucleotide, flavin dinucleotide, coenzyme A, adenine polyphosphate, thiamine pyrophosphate, pyridoxal phosphate, and cytochrome c. Biotin and folic acid are present in lesser amounts. These coenzymes appear to be firmly bound to their appropriate apoenzymes, since they resist leaching out when the enzyme particles are washed exhaustively by repeated centrifugation and suspension in cold saline solution.

Several enzymes are known to exhibit vastly different requirements for added coenzyme depending upon whether they are present in the organized cyclophorase complex or are obtained as isolated, soluble proteins. Thus the soluble malic and citric enzymes are isolated as apooxidases which are completely inactive unless supplemented with diphosphopyridinenucleotide (DPN) and triphosphopyridinenucleotide (TPN), respectively (Straub, '42; Adler et al., '39). The same oxidases in the cyclophorase complex show no requirement for added pyridine nucleotide (Huennekens and Green, '50a, b). The isolated malic and citric apooxidases function maximally only when the ratio of coenzyme to enzyme has a value well in excess of 10^2. In the cyclophorase system the ratio of coenzyme to apoenzyme appears to be of a much smaller order of magnitude.

The concept of bound coenzymes in a chemical mosaic raises some difficult problems of mechanism. In the interaction between two soluble pyridinoprotein enzyme systems leading to the oxidation of the reductant of one system by the oxidant of the second system the dissociable prosthetic group provides a means of linking the two enzymes by shuttling back and forth between the two. The cyclophorase complex catalyzes similar interactions between its constituent pyridinoprotein enzymes. If the pyridine nucleotide is bound to its appropriate apooxidase, it becomes difficult to visualize how a coenzyme-linked reaction between two conjugated enzymes can take place. The coenzyme no longer has the potentiality of a shuttle. We must anticipate some new principle of mechanism operative within the cyclophorase complex.

OXIDATIVE PHOSPHORYLATION

Since early 1940 it has been known that the aerobic oxidation of various metabolites in tissue homogenates is accompanied by the esterification of inorganic phosphate. Belitzer and Tsibakova ('39), Kalckar ('39a, b), Cori ('41), Colowick et al. ('41), and Ochoa ('44) are responsible for the pioneer investigations in this field. Studies in our laboratory in 1947 disclosed that the phenomenon of oxidative phosphorylation was a characteristic of the cyclophorase complex of enzymes, and indeed constituted the most diagnostic attribute of the complex (Cross et al., '49; Green et al., '49). Every oxidative step of the citric acid cycle was shown to be accompanied by the esterification of inorganic phosphate. In addition, oxidative phosphorylation was shown to accompany the oxidation of L-glutamate, L-proline, and DL-β-hydroxybutyrate. The P/O ratio (molecules of inorganic phosphate esterified per atom of oxygen absorbed) approximated three for all steps except the succinic step, the ratio for which approximated two (Cross et al., '49).

This phenomenon of oxidative phosphorylation has provided the most convincing and compelling evidence for the cyclophorase concept. If, as it was argued by some, the cyclophorase complex was no more than a chance mixture of a large assortment of separate enzymes, what explanation can be invoked to account for the fact that a given oxidase should exhibit a property when present in the mixture that it lacks when separated from the mixture? The emergence of a new property indicates a real difference in the nature of the enzyme in question.

Limitation of space precludes a full discussion of the experimental evidence bearing on oxidative phosphorylation which various laboratories have contributed. The significant features may be summarized as follows:

1. There is no evidence that the substrates become esterified, e.g., in the form of acyl phosphates, during oxidative phosphorylation (Cross et al., '49).
2. There is circumstantial evidence that the oxidation-reduction coenzymes are concerned in the mechanism of oxidative phosphorylation (Green et al., '49; Lehninger, '49).
3. The first product or products of oxidative phosphorylation appear to be a highly labile phosphoric ester (gel P) which readily decomposes to liberate inorganic phosphate (Cross et al., '49).
4. All the substrates whose oxidation leads to oxidative phosphorylation give rise to gel P.
5. Inorganic phosphate is necessary for maximum rate of oxidation of metabolites in the cyclophorase system. In absence of inorganic phosphate the rate declines to less than one-third the maximum, but never falls to zero (ibid.; Ochoa, '44).
6. Dinitrophenol appears to interfere not with the esterification of inorganic phosphate, but rather with transfer of high-energy phosphate gel P to phosphate acceptor systems. In presence of dinitrophenol, gel P is rapidly broken down to inorganic phosphate (Loomis and Lipmann, '49; Teply, '49).

The available experimental evidence may be integrated in the form of the following working hypothesis which may assist in an appreciation of the underlying differences between the properties of certain oxidase in the organized and soluble states.

1. substrate + oxidase $\xrightarrow{-2e}$ oxidized substrate-reduced oxidase.
2. oxidized substrate-reduced oxidase + phophate → phosphate-reduced oxidase + oxidized substrate.
3. phoshate-reduced oxidase + adenosinediphosphate → reduced oxidase + adenosinetriphosphate.

The oxidation leads to a compound between oxidized substrate and reduced oxidase. The link between the two has the character of a high-energy bond. Inorganic phosphate replaces the oxidized substrate and a high-energy phosphate link is formed with the reduced oxidase. Gel P corresponds to this hypothetical compound. According to this conception, inorganic phosphate is not necessary for the primary reaction. However, in the absence of inorganic phosphate, the primary complex is stabilized and the over-all reaction velocity becomes limited by the spontaneous rate of breakdown of this complex. A similar mechanism can readily be constructed for the interaction of reduced oxidase with the next coenzyme in the respiratory chain. In this instance, the reduced oxidase now serves as substrate for the next oxidase in line.

The essential point, according to the above speculation, is that oxidative phosphorylation depends upon the formation of a high-energy bond between the substrate and the oxidizing enzyme. Once this is formed, inorganic phosphate can replace the substrate, and a high-energy phosphate bond is created. It would appear that the isolated oxidases catalyze an oxidation which either does not lead to the formation of a compound between oxidized substrate and oxidizing enzyme, or which leads to a compound whose life term is of such short duration that it cannot be used in subsequent reactions.

TRANSFORMATION OF ENZYMES

Studies on the cyclophorase system have pointed up a phenomenon, the validity of which has long been suspected by enzyme chemists, viz., that enzymes are modified or transformed in the process of isolation. The properties of a fair number of enzymes are known both when these enzymes are part of the cyclophorase complex and when they are studied as discrete, soluble proteins. The comparison of properties has revealed extensive differences in some though not in all cases. Mention has already been made of the differences between oxidases at the two levels of organization with respect to the ability to carry out oxidative phosphorylation, and to the extent of dissociation of the prosthetic group. Huennekens ('51) has made a systematic comparison of the properties of the L-malic oxidase in the state in which added coenzyme is not required (conjugated) and in the state in which there is a requirement (dissociated). Profound differences were observed in the reaction kinetics, and in the susceptibility to various reagents as shown in table 1.

Evidence is also accumulating from other areas of investigation that the properties of enzymes may be modified in the process of isolation. For example, in E. Cohn's laboratory, Surgenor (Cohn et al., '51) has isolated catalase in the form of a complex which differs in various respects from catalase prepared by classical procedures. The Coris ('45) have isolated mammalian starch phosphorylase in two forms — one of which requires adenosine-5-phosphate (amp) for activity, while the other not only does not require such addition, but does not even contain amp in a bound form. The conversion of the one form to the other can be brought about by a specific enzyme. There can be little doubt now that transformation of modification of enzymes does indeed take place. The problem is to determine which enzymes undergo transformation and of what character.

TABLE 1

PROPERTY	STATE OF ENZYME	
	Conjugated	Dissociated
Requirement for added pyridine nucleotide	No DPN required	DPN required
Effect of 2,4-dinitrophenol, capryl alcohol and arsenite	Inhibited	Not inhibited
pH Optimum	7.0–8.0	9.5
Effect of oxalacetate	Not inhibited	Inhibited
Substrate concentration, saturation	8×10^{-3} *M*	140×10^{-3} *M*

(Huennekens, '51)

MITOCHONDRIA

During the past 10 years, Claude at the Rockefeller Institute, and Hogeboom and Schneider, have been carrying on systematic investigations dealing with the nature and separation of the particulate elements of the cell. They devised a practical method of sorting out the different particle types which depends upon applying graded increases in centrifugal speeds to cell homogenates suspended in strong sucrose solution (Claude, '41, '43, '47–'48; Hogeboom et al., '46, '48; Hogeboom and Schneider, '50c). They soon recognized that there were only limited numbers of particle types which were sharply distinguishable one from the other by specific enzymatic properties and chemical characteristics. The insoluble residue obtained by centrifuging homogenates has a somewhat unprepossessing appearance and consistency. It is little wonder that enzyme chemists have traditionally regarded this insoluble fraction as a hopeless mess — a hodgepodge of enzymes, from which one had to emerge with the greatest expedition. The work of Claude, Hogeboom, Schneider, and others has done a great deal to overcome this bias toward the particulate fraction, and to bring conviction that there is just as much uniformity and homogeneity in purified particulate fractions as is to be found in many of the crystalline enzymes.

Some studies of the Rockefeller group were directed toward the characterization of the enzymatic properties of the large granule or mitochondrial fraction, and the submicroscopic, microsomal fraction. Between 1946 and 1950 these studies, owing to the limited number of satisfactory assay systems, were somewhat restricted in scope. Cytochrome oxidase, succinic oxidase, and DPN- and TPN-cytochrome reductases were the only enzymes covered in their survey (Hogeboom, '49; Hogeboom and Schneider, '50a, b). Criteria were set up by which a decision could be reached on whether any enzyme was localized in any one cell fraction. On the basis of this criterion, DPN-cytochrome reductase was localized in the microsomal fraction, isocitric oxidase in the soluble fraction, and succinic oxidase in the mitochondria. No conclusions could be drawn about the localization of TPN-cytochrome reductase. As far as their data were concerned, there was little to choose between mitochondria and microsomes with respect to the above enzymatic activities. The tests applied were not designed to measure reaction sequences, and consequently the decisive differences between the mitochondria and microsomes which were earlier recognized in the cyclophorase studies, were not revealed. There was one discrepancy in the data reported which raised doubts as to the reliability of the criteria set up for determining localization. The isocitric oxidase was ruled out by Hogeboom and Schneider as a constituent of the mitochondria, although the studies of all other workers in the field have shown the activity of the isocitric oxidase in mitochondria to be on a par with that of the succinic oxidase. In their most recent publication Hogeboom and Schneider ('51) maintain that the mitochondria carry out the reactions of the citric acid cycle (oxalacetic oxidase), and it must be inferred therefore that they no longer regard their data on the concentration of isocitric oxidase in nonmitochondrial fractions as a bar to assigning this oxidase a place in the mitochondrial galaxy of enzymes. The work on the cyclophorase system led to the concept of an integrated enzyme complex of at least a hundred enzymes associated with some structural unit in the particulate fraction. The work on the fractionation of cellular particulates led to the recognition of structural units which clearly played a significant role in cellular respiration as evidenced by the presence of one or two key enzymes. One group was casting for a structural unit, while the other was searching for an enzymatic function. The marriage of these two lines of investigation was inevitable, and was happily consummated in 1949–1950. Lehninger and Kennedy at the University of Chicago ('49), and Schneider and Potter at the University of Wisconsin in the same year independently announced that mitochondria, free from other cellular components, carried out the reactions of the citric acid cycle and fatty acid oxidation. This identification of the structural unit of the cyclophorase complex with the mitochondrion was doubly gratifying to our laboratory. On the one hand, the association of an enzymatic unit with a well-recognized and accepted structural unit carried far more conviction among enzyme chemists than had the evidence of a purely enzymatic character. On the other hand, the evidence presented by Lehninger and Kennedy, and Schneider and Potter for considering the mitochondrial unit as the structural housing for the enzymes of the citric acid cycle and ancillary reactions could be valid only if the concept of a complex of enzymes was a valid one. If one were to regard mitochondria as a mixture of some hundred different kinds of particles of similar size and shape, each bearing a separate enzymatic function, the identification would be meaningless. The assumption in the evidence was tacit that each particle contained the full complement of all the necessary enzymes, and that the unit was not the single enzyme, but a complex of a large number of enzymes.

Leuthardt and his group in Zurich inaugurated a series of studies in 1948 which have shown that mitochondria catalyze synthetic reactions such as the synthesis of citrulline from ornithine, ammonia, and carbon dioxide (Leuthardt and Müller, '48) — a reaction which was discovered by Cohen and Hayano ('47) in washed residues of rat liver, though not referred specifically to mitochondria. More recently, Kielley and Schneider ('50) have confirmed the localization of the hippuric synthesizing system in mitochondria.

In our laboratory, Harman ('50a, b) has brought to bear several additional lines of evidence bearing upon the site of cyclophorase activity in mitochondria which may be summarized as follows:

1. There is a close parallelism between cyclophorase activity and the integrity of mitochondria. Procedures and reagents which lead to the dispersion or modification of mitochondrial structure also lead to the loss of cyclophorase activity.

2. There is a good correlation between the distribution of mitochondria and cyclophorase activity in fractions from tissue homogenates.
3. Cyclophorase activity cannot be correlated with nuclei or microsomes.
4. The mitochondria carry on oxidative phosphorylation, the most characteristic attribute of the cyclophorase system.

Paul and Sperling (unpublished observations) have made a comparative study of cyclophorase activity in the breast muscles of different birds. Excellent correlation was observed between the cyclophorase activity of extracts of these muscles and the density of mitochondria. White breast muscle is consistently low both in cyclophorase activity and mitochondria, whereas red breast muscle is consistently high in both respects.

Hogeboom and Schneider during the past two years have undertaken a systematic and critical examination of the enzymatic composition of mitochondria. They have fully confirmed the important discoveries of the Lehninger and Potter laboratories. It is gratifying to note that their list of the enzymes in mitochondria (Schneider and Hogeboom, '51) coincides exactly, except for some minor details, with the list of enzymes in the cyclophorase complex (Green et al., '47).

THE COMMINUTION OF MITOCHONDRIA

When a mitochondrial suspension is exposed to the shearing forces of the blades of a Waring Blendor, the particles are gradually reduced in size below the level of microscopic visibility though their particle weight is still several orders of magnitude larger than that of average soluble proteins, and they are readily sedimented when centrifuged at 17,000 × gravity (Still and Kaplan, '50). The products of comminuted mitochondria are indistinguishable operationally from the microsome fraction which is found in all cell homogenates after removal of mitochondria. As long as microsomes are defined in terms of size and sedimentation characteristics, there is no way of distinguishing between preformed microsomes, if such exist, and microsomes derived by breakdown of mitochondria. The enzymatic data are as yet incomplete on this score, but the available evidence does not encourage the view that what we may call the apparently preformed microsomes show essentially different enzymatic properties from those known to be derived from mitochondria. Some differences in chemical composition between mitochondria and "preformed" microsomes have been regarded as evidence for the separate origin of microsomes (Chantrenne, '47). In view of the fact that the comminution of mitochondria is known to be accompanied by the loss of enzymes, and the unmasking of destructive enzymes, the significance of small differences in chemical composition is hard to evaluate.

The microsomes obtained by fragmentation of mitochondria from rabbit liver or kidney exhibit many of the original enzymatic activities of the parent unit (Still and Kaplan, '50). However, the more complex enzymatic sequences such as fatty acid oxidation, oxidative phosphorylation, and the synthetic reactions appear to have been lost. This consequence is not necessarily true for mitochondria from all tissues. Only in part may the loss of activity reflect loss of organization. The most important factor is probably the loss of enzymes or components essential for the more complex reaction sequences.

The studies of Kaplan and Still ('50) and Harman ('50a, b) have pointed up that enzymes show varying tendencies to be detached or separated out from the mitochondrial bodies. Certain enzymes can be leached out completely by continued washing of mitochondria in cold saline solution. A whole spectrum exists between the readily detachable enzymes at one extreme and enzymes which under no circumstances can be detached at the other. There is therefore no sharp line of demarcation between enzymes which are held in the complex by strong bonds, and those held by relatively weak bonds. The differences are merely quantitative in nature. It is thus impossible to decide where chemical linkages end and absorption begins.

This aspect has considerable bearing on the question of the localization of certain enzymes. It is not sufficient to carry out a fractionation of homogenates under arbitrary conditions, and decide on the basis that more of a particular enzyme is in the microsome or soluble fraction than in the mitochondrial fraction, that this enzyme is not a normal constituent of the mitochondria. If the fractionation procedure is carried out in a medium which favors the leaching out of the particular enzyme from the mitochondria, the distribution of enzyme may throw no light on the physiological locale of the enzyme. Until more is known of the forces by which the constituent enzymes of mitochondria are held together, and of the effect of the suspending medium on these forces, many of the studies now directed at the quantitative localization of enzymes would appear to be premature. The complete absence of certain enzymes in mitochondria or the exclusive association of certain activities with mitochondria may permit of a decisive answer. Where there is a spread of distribution, decisions on locale must presuppose the very knowledge at which the experiments are aimed.

DO MITOCHONDRIA HAVE MEMBRANES?

A vast number of metabolites, such as coenzymes, inhibitors, and salts have been tested with mitochondrial enzymes. As yet no direct evidence has appeared that mitochondrial enzymes are inaccessible to any of the substances tested. The fact of selective permeability is the usual basis for postulating a membrane for cellular elements. Since mitochondria apparently are permeable or accessible to any type of dissolved molecule,[2] one may well inquire if there is any basis or reason for postulating a mitochondrial membrane. No doubt some sort of interface exists at the point of contact of the mitochondrion with the suspending liquid. But is there any need to invoke something other than the interface which exists, let us say, at the point of contact of a virus particle with the suspending fluid? The tendency of mitochondria to swell or shrink as the ionic strength of the medium is varied, has been interpreted by some as evidence that mitochondria are little cellules which, like red blood corpuscles, contain a fluid interior enclosed by a delimiting semipermeable membrane (Dalton et al., '49). However there are complex proteins such as gelatin, myosin, and viruses which register precisely the same type of osmotic behavior as mitochondria, without such behavior being interpreted in terms of fluid interior and membranes.

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[2] Harman ('50a) with the use of radioactive sodium and potassium salts has shown that mitochondria are freely permeable to these ions, and that these ions are neither concentrated selectively within nor excluded from the mitochondrial particles.

Dalton et al. ('49) have recently adduced electron microscopic evidence of the presence of membranes in mitochondria. Considering the drastic means employed to prepare specimens for examination in the electron microscope, and the difficulty of interpreting the photographs of mitochondria exposed to the electron beam, it would appear to be somewhat hazardous to base claims for a mitochondrial membrane exclusively on such evidence. If there is a mitochondrial membrane, some property should be demonstrable, which is interpretable uniquely in terms of a membrane. As yet no such property has been reported.

There are at least two alternative pictures of mitochondrial structure. One can conceive of the mitochondria either as a highly polymerized macromolecule like a virus particle, or as a little corpuscle-like cellule with the individual enzymes dissolved in the fluid interior. That mitochondria on comminution give rise to fragmented particles which still contain many of the enzymatic activities of the original unit would appear to favor the polymerized macromolecule hypothesis. It is of course not excluded that the true state of mitochondria might be somewhere between the two possibilities enumerated.

ASSAY OF ENZYMES

The fact that a mitochondrial oxidase may show different properties from those of the same oxidase in soluble form has important implications for the assay of certain enzymes. For example, the citric oxidase in the cyclophorase complex is conjugated with respect to its prosthetic group (Huennekens and Green, '50a, b). It remains to be determined how the rate with which it reacts with triphosphopyridinenucleotide added to the preparation compares with the velocity of oxidation of citrate by molecular oxygen. If the citric oxidase content of mitochondria is assayed by the procedure generally employed in testing the soluble citric oxidase, an erroneous estimate may be arrived at. When the citric oxidase is tested for, under the conditions for demonstrating maximal cyclophorase activity, its level in the mitochondrion compares very favorably with the amounts in other fractions. Many of the difficulties and uncertainties experienced in studies of localization based exclusively on arbitrary assay procedures can be resolved by invoking the data on the properties of enzymes within and without the cyclophorase complex. The citric oxidase in the mitochondrion is conjugated, requires inorganic phosphate, and carries on oxidative phosphorylation; whereas, the same oxidase in the microsomal fraction is generally dissociated, does not require inorganic phosphate, and does not carry on oxidative phosphorylation (ibid.; Cross et al., '49). Evidence of this nature permits an unequivocal assignment of certain enzymes to the mitochondria, even though on the basis of the localization data, these enzymes may not be concentrated in mitochondria.

There is the reverse difficulty in assaying certain enzymes such as fatty acid oxidase (also called octanoic acid oxidase) and condensing enzymes. Since the activity of these enzymes depends upon other simultaneous processes, and a complex series of interactions, there is no way at present of testing for these enzymes in fractions other than the mitochondrial fractions. There may well be present some of these enzymes in the submicroscopic or soluble fractions, but they cannot be assayed for under the test conditions which apply to the cyclophorase system.

The desire to accumulate information of a quantitative nature as to the relative amounts of various enzymes in different cell fractions is indeed a legitimate one. But account must be taken of the differences in properties of enzymes in the organized and soluble states, and of the different coenzyme requirements of these enzymes. In the cyclophorase system, the citric oxidase catalyzes the oxidation of citrate to α-ketoglutarate with concomitant esterification of inorganic phosphate. The limiting factor in the over-all velocity of this oxidation appears to be the rate of release of phosphate from the phosphorylated enzyme. In presence of dinitrophenol, a reagent which speeds up the release of phosphate, the rate of oxidation of citrate can be doubled (Cross et al., '49). It becomes a somewhat arbitrary procedure to compare the relative activities of the citric oxidase in mitochondria and microsomes, if no account is taken of limiting factors other than the amount of oxidase.

In our laboratory, Mahler and Huennekens ('52) have shown that the lactic oxidase of the rabbit liver cyclophorase-mitochondrial system is D-specific, and requires no addition of pyridine nucleotide. When the mitochondria are comminuted, the lactic oxidase now is L-specific and dependent upon pyridine nucleotide for activity. How the balance-sheet approach to the localization of enzymes would operate in an instance of this kind is difficult to imagine.

PREPARATION OF THE CYCLOPHORASE SYSTEM

While the identification of the structural unit of the cyclophorase system with the mitochondrion appears to be reasonably certain, cyclophorase system is not a synonym for mitochondria. One must not forget that the terms cyclophorase and mitochondrion are concerned with two quite separate aspects of the same phenomenon. Cyclophorase refers to the functional and enzymatic aspects of the unit; whereas, mitochondrion refers to the structural and morphological aspects. Methods may be devised for obtaining mitochondria free of other cellular elements, but the resulting preparation, although of value from the morphological point of view, may be valueless from an enzymatic point of view. The isolation of a homogenous component is not synonymous with the isolation of that component in its most active biological state. Purity in enzyme chemistry has significance only when related to biological activity. One must apply such criteria in evaluating the different methods for the preparation of the cyclophorase-mitochondrial system.

In the early experiments with kidney cortex cyclophorase, where the emphasis was placed on maximum enzymatic activity, the complex was prepared by homogenizing the tissue (diluted 1:5 with 0.9% potassium chloride) in a Waring Blendor and repeatedly washing the residue obtained by short centrifugation at 3000 × gravity in 0.9% potassium chloride solution. All operations were carried out at 0°C. and completed within 45 minutes after the tissue was removed from the experimental animal (Green et al., '48). The residue contained no whole cells or microsomes. Microscopic examination showed mitochondria and nuclear fragments as the principal constituents (Harman, '50a, b). Such a crude preparation was uniformly active and relatively stable for some hours at 0°C.

The same procedure applied to liver proved far less satisfactory, and only recently has the difficulty been resolved. When liver cells are ruptured in the Waring Blendor under precisely the same conditions as for kidney cells, the mitochondria of the former appear to be extensively damaged; whereas, the mitochondria of the latter are relatively unaffected. This is probably not due to an inherent difference in the fragility of mitochondria from these two sources, but rather to a protective factor which is unique to kidney cortex. The tubular epithelial cells are enclosed by a connective tissue sheath (basement membrane)[3] which remains intact during the homogenization in the Blendor (Harman, '50a, b). The sheath is sufficiently elastic to permit complete fragmentation of all the cells within it, but yet rigid enough to shield the mitochondria liberated from the cells from the full shearing action of the rotating blades. This protective effect of an enclosing sheath does not apply to the liver cells. The remedy in the modified preparation of active liver cyclophorase is to reduce the speed of the rotating Blendor blades from 17,000 to about 12,000 rpm (Beinert et al., '51). At this speed, however, and for the usual homogenization time (45 seconds), not all the cells are completely fragmented. The use of sucrose solutions introduced by the Claude school has proved very useful in the preparation of liver cyclophorase. The liver is homogenized in 5 volumes of 8% sucrose. The unfragmented cells, nuclear elements, and red blood corpuscles are removed by centrifugation at ca 1000 × gravity. The supernatant fluid is diluted with three volumes of 0.9% potassium chloride and the mitochondria are then sedimented at 3000 × gravity. This leads to a preparation, microscopic and histological examination of which reveals essentially only mitochondria (Q_{O_2} of 40 — 60). This type of preparation is not applicable to kidney, since the enclosing basement sheath gives sedimentation characteristics to the mitochondria which they would not have as free mitochondria. As a result, the great bulk of the mitochondria come down in the first nuclear fraction, and the yield in the second fraction is inadequate for enzymatic studies. No simple method has yet been devised for preparing the cyclophorase system from kidney in good yield without contamination of the mitochondria by other cellular elements. While it is relatively simple to obtain homogenous mitochondria in limited amount from kidney cortex, the yields are not adequate for enzymatic studies.

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[3] In order to avoid the confusion which the term "membrane" may lead to in view of the previous discussion of the mitochondrial membrane, the term "sheath" is used although histologists refer to this structure as basement membrane.

There are as yet insufficient data to determine how the activity of the standard mitochondrial preparations of Hogeboom, Schneider, and others compare with the cyclophorase preparations made in our laboratory. Using the Q_{O_2} for succinic oxidase is not an adequate basis for comparison, since this is one of the few sturdy enzymes in the complex whose activity is not greatly influenced by the mode of preparation. The effects of the suspending medium and the electrolyte content on the ultimate enzymatic activity have yet to be evaluated quantitatively. Criteria such as the rate of fatty acid oxidation, or of hippuric acid synthesis and the magnitude of the P/O ratio provide a much more reliable index of the activity of a cyclophorase preparation than the rate of oxidation of some metabolite such as succinate or citrate, which can be oxidized readily even in preparations that have lost the original capacity for oxidative phosphorylation.

DISCUSSION

CHAIRMAN CARTER: This paper is now open for discussion.

GROSECLOSE: I should like to ask Dr. Green if he has any ideas about the origin or reproduction of these mitochondria.

GREEN: No, I have made no contribution to that problem, though I am aware of speculation that they may be self-duplicating units.

BOELL: There appear to be three views on the relationship of microsomes and mitochondria. The first, that they are unrelated, discrete units in the cell, can, I think, be dismissed summarily. The view that the microsomes are disintegration products of mitochondria has already been expressed. A third view, which stems largely from the work of Brachet and his collaborators, is that the microsomes represent steps in the elaboration of mitochondria, or that microsomes differentiate, if we may use that term, into mitochondria. The evidence for this is based largely on the fact that a series of populations of small granules can be isolated by differential centrifugation. Additional evidence for this view is the finding that the enzymatic potentialities of mitochondria undergo considerable change during embryonic development as shown by a progressive increase in the amount of enzymically active protein in association with the mitochondria.

GREEN: I think that, of the three possibilities that you mention, we are in accord that one of these, viz., the possibility that microsomes and the mitochondria are completely unrelated, seems to be definitely out.

The other two possibilities would appear to be variants of the same. You might think of the mitochondrion as really a polymerized molecule with repeating units, each of which has the character of a microsome, if you like. Then the break-up of the mitochondrion is attended by the liberation of the constituent microsomal units. In that sense, microsomes are normal constituents of cells.

I would be willing to agree with you, that the mitochondria may be cleaved in a regular way into microsomes.

DOUNCE: You did not mean to imply that there is no such thing as microsomes in the cell, did you? It seems to me that this question could be investigated optically.

GREEN: How would you do it optically?

DOUNCE: By directly observing, with dark field illumination, particles which are much smaller than the mitochondria. Furthermore, by a laborious method you might be able to find out how many mitochondria there are in a given volume or in a given weight of tissue and then relate that value to the number of cells. The number of cells in the same amount of tissue could be estimated by counting methods or by analysis for DNA. Thus it would be possible to calculate whether there are enough mitochondria in the cells to account for the insoluble protein. Did you mean to imply that there are no particles in the cell between the nucleus and the cell membrane, other than the mitochondria and some other large granules?

GREEN: That is not what I meant. I am merely pointing out that there is no compelling evidence, as far as I am concerned, that the enzymatic properties of microsomes differ from those of mitochondria; and, in the absence of such evidence, one is not justified in regarding them as two completely separate entities. There may be preformed microsomes, or these may arise as the result of the cleavage of mitochondria. There is not sufficient evidence to choose between the two possibilities. I would not distinguish mitochondria from microsomes in the same way that I would distinguish nuclei from mitochondria; the latter two have completely different spectra of enzymes. I do not know whether there are particles intermediate in size between the nucleus and mitochondrion other than the microsomes.

DOUNCE: Don't you agree that there is a difference in enzymatic composition between mitochondria and microsomes? Cytochrome oxidase for instance is not found in the microsomes, is it?

GREEN: I do not know how that concept has arisen, since Keilin, in his classical work on cytochrome oxidases, used preparations which were exclusively microsomal in nature.

DOUNCE: The particles obtained from muscle are different. In a cell like the liver cell, I think one finds all the cytochrome oxidase in the mitochondrial fraction, the microsomal fraction being essentially free of this enzyme.

GREEN: You can grind up mitochondria so none are left. The residue from this operation consists of microsomes, which contain cytochrome oxidase with undiminished activity.

DOUNCE: I understand that. But I also understand that you can actually fractionate liver cell into mitochondria and the microsomal fraction. The microsomal fraction does not have cytochrome oxidase and the mitochondrial fraction does.

GREEN: As far as my own personal experience is concerned, I have yet to see a microsomal fraction from any tissue that does not contain cytochrome oxidase.

DOUNCE: I am quoting from the literature. According to Schneider and a number of others, you can account for 99% of the cellular activity of cytochrome oxidase in the mitochondrial fraction. Furthermore, the microsomal fraction is very appreciable in quantity.

GREEN: I think you yourself have pointed out what is probably the answer to that discrepancy, viz., that mitochondria are self-contained units and are not subject to the destructive actions of all the degradative enzymes; whereas, the microsomes may be severely damaged during their isolation from homogenates.

It is interesting that in only these tissues which have relatively little proteolytic action is the microsomal fraction respectable, as far as enzymatic activities are concerned. In liver and kidney cells, the preformed microsomal fraction is usually weak as far as enzymatic activities are concerned, owing to destructive processes.

DOUNCE: There is one more piece of evidence that has something to do with whether there are small particles in the cell besides the mitochondria, which correspond to the isolated microsomes. This is the work, dealing with centrifugation of whole cells, that has been carried out by Claude. He centrifuged cells and obtained a considerable stratification of the material within these cells. Although I do not remember exactly the order of sedimentation, I think the glycogen went to the bottom and the nucleus rested on that, with the mitochondria in the next layer (going up) with a microsomal layer above them, and finally a clear layer on top. I may not have had the order quite correct. But I wonder if that furnishes some sort of indication that finer particles exist in the cell together with mitochondria? It looked fairly good from the picture.

GREEN: How was he able to determine that the layer above the mitochondria consisted of microsomes as opposed to other types of particles?

DOUNCE: He carried out some staining. As I recall it, he could distinguish the microsome layer since microsomes stain intensely with certain dyes on account of their high nucleic acid content. I am sorry I do not remember the exact details.

STREHLER: I would like to ask Dr. Green whether he has any opinions on the question of how these cytochrome-coupled oxidative phosphorylations are achieved, especially in the later stages of hydrogen transport when one gets down to the level of reduction of cytochrome c. Since most of the oxidative phosphorylations that we know something about seem to involve two electron transfers and the cytochrome system seems to be a one-electron transport system, I wonder whether he feels that there is a possibility of intermediate two-electrons transporting coenzymes acting between the different cytochromes, with the cytochromes serving merely as way stations in the transport. Is there any information bearing on this point?

GREEN: The only information that is available comes from the work of Slater, who has shown that when oxygen is replaced by cytochrome c as electron acceptor the same P/O ratio is found.

If we assume that Slater's conclusions are correct, then it would follow that the coenzymes involved in oxidative phosphorylation cover only a span of some 600–800 mv between the potential of the hydrogen electrode and the potentials of the cytochromes. The span of some 600 mv between the cytochromes and molecular oxygen would not be covered by any coenzymes. The question arises then how it is possible to get a very high efficiency if approximately half the total potential drop is neglected, as it were. In principle it may be more convenient, though it is not necessary, to space the coenzymes in such a way as to cover the entire scale of potential between the substrate and oxygen. The driving force of oxygen is a factor which cannot be neglected. In absence of oxygen, the equilibrium point of the reaction involving oxidative phosphorylation may be far from completion. The driving force of oxygen can be used to push these equilibria far to the right which is then tantamount to having a whole series of coenzymes with potentials extending to that of the oxygen electrode.

WILDE: In relation to a possible membrane, would you care to discuss the mechanism of the osmotic construction of the over-all reactions? Do you feel that there are certain denaturations going on, or is there any evidence of subgroupings or reactions that are interfered with osmotically?

GREEN: Are you referring to the fact that mitochondria in different types of media swell or shrink? Sometimes the mitochondria swell to the point where the outlines of individual units disappear. This swelling and shrinking is characteristic of many proteins as, for example, myosin and gelatin.

Wilde: The question is, does that denature or affect all the subreactions or just the over-all picture?

Green: I think mitochondria can shrink and swell to a limited extent. But, beyond a certain point an irreversible process sets in. Cross linkages are probably broken and new ones formed irreversibly.

Carter: I would like to ask Dr. Green if it is necessary to postulate that the measured redox potential is representative of conditions in the cell. Might the coenzymes not extend, when they are bound to the protein, over an entirely different voltage from those that are measured when we used dissociated coenzymes in 50–50 ratio against a calomel electrode?

Green: I think your point is very well taken. There is very clear evidence that, when coenzymes are linked to proteins, their oxidation-reduction potential tends to be more positive. But I think we know the maximum extent of this rise in potential. It is not much more than some 100 mv; whereas, a shift of some 500 mv would be needed to cover the full potential scale. The cytochromes would have to show this shift in oxidation-reduction potential since they are the most positive coenzymes.

Carter: To what extent can you cite evidence for organized glycolytic enzymatic actions?

Green: We have found that the enzymes involved in the fundamental oxidoreductions of glycolysis are present, at least in part, in mitochondria.

I was interested to hear Dr. Dounce say that the triphosphoric enzyme is also present in nuclei. Thus the enzyme has been found (a) in solution, (b) in the mitochondria, and (c) also in nuclei. The amount that is present in mitochondria is not very high. The triosephosphoric enzyme of mitochondria may not be concerned with glycolysis at all, because other glycolytic enzymes are absent from mitochondria.

Ducoff: As I understand it, the coenzyme must be activated before it can go into synthesis such as fatty acid synthesis. At the same time these coenzymes are used in the citric acid cycle presumably for production of high-energy phosphate. Is it possible to add so much citric acid cycle intermediate that you swamp things like fatty acid metabolism? That is, would there be competition between the citric acid oxidase and the fatty acid oxidase for the same coenzymes?

GREEN: There may be some competition in the sense you have indicated though I doubt whether this would be a serious problem. You may recall the formation of succinyl-enzyme during the oxidation of α-ketoglutarate and the replacement of the succinyl group by a phosphoryl group, that phosphate must be removed for the reaction to keep on going.

Ordinarily this discharge depends upon the interaction of the phosphoryl group with ADP. If this possibility is not realized, there are other means of removing that phosphate which do not involve the utilization of phosphate-bond energy. Phosphoryl-enzyme can also be broken down hydrolytically. High-energy phosphate is being continuously generated. If there is no available acceptor system the bond energy is wasted. It is like a car that is idling.

KRAKAUR: Are there any other substances besides dinitrophenol and gramicidin which will inhibit oxidative phosphorylation?

GREEN: A variety of substances. Atabrine has been shown to work in this manner, and I understand some of the oxidation reduction dyes like methylene blue also work well in this way.

KRAKAUR: Dissociated process?

GREEN: Yes. This so-called dissociation of oxidative phosphorylation, I think, needs a word or two of comment. There is an impression that dinitrophenol separates oxidation from phosphorylation, but there is evidence which does not sustain that interpretation. The results with radioactive phosphorus show that phosphorus can be taken up in the presence of the dinitrophenol and it would appear that something like the following is taking place: let us return to the succinyl-enzyme formed during oxidation of α-ketoglutarate. We replace the succinyl group by phosphate and phosphoryl-enzyme is formed. That is probably what we have called gel P. Dinitrophenol has the property of splitting out inorganic P from gel P. In other words, high-energy phosphate, instead of being used, is simply released by dinitrophenol before any work can be done; the bond between the enzyme and the phosphoryl group is broken, and inorganic phosphate is liberated.

Dinitrophenol does not prevent the formation of phosphoryl enzyme but rather dephosphorylates the complex as quickly as it is formed.

DOUNCE: How do you know it is down to that enzyme and then into something else?

GREEN: We do not mean necessarily that enzyme, but an enzyme concerned with the formation of high-energy phosphate; because we can show this by means of radioactive phosphorus.

If you carry out oxidative phosphorylation in the presence of radioactive inorganic phosphate, you find an incorporation of phosphate in the mitochondria in a form which is not removed from the particles by washing at low temperatures. On addition of dinitrophenol, the incorporated radioactive phosphate is released into the medium and can then be removed from the mitochondria; this result suggests that the phosphate thus liberated was previously bound.

CARTER: Would you care to comment on the nature of your succinyl enzyme?

GREEN: Yes. It was demonstrated as follows in our laboratory by Dr. Sanadi. You may know that in the Lipmann system, ATP in presence of acetate, CoA and sulfanilamide yield acetyl sulfanilamide. Recently this process has been analyzed and it has been shown that acetyl CoA is formed as an intermediate. Acetyl CoA then reacts with sulfanilamide to form acetyl sulfanilamide.

In a very similar way, the α-ketoglutaric oxidase yields succinyl sulfanilamide in the presence of CoA and the Lipmann system. First, succinyl CoA is formed and then succinyl CoA reacts with sulfanilamide to form succinyl sulfanilamide.

This reaction with sulfanilamide is not specific for acetate. It just happens that, historically, acetate was used, but this condensation reaction appears to be general for acyl CoA.

CARTER: Have you been able to show the binding of the succinyl to the enzyme?

GREEN: In this system of Sanadi, there are two enzymes. The first enzyme generates succinyl CoA; the second catalyzes the condensation of succinyl CoA with sulfanilamide. CoA is oscillating between the two enzymes.

Thus if we are forming succinyl sulfanilamide, we must be forming a succinyl CoA as an intermediate.

CARTER: But have you been able to show that the enzyme actually accepts succinyl?

GREEN: Yes. Dr. Sanadi has recently succeeded in isolating succinyl CoA as the product of the interaction of α-ketoglutaric oxidase with CoA.

CARTER: Is this different from the pathway of acetate? I mean, was phosphate necessary to raise acetate to an acetyl phosphate so that you would get CoA, acetyl CoA?

GREEN: There are two ways of making active acetate. One can start off with a soluble pyruvic oxidase and make acetyl CoA by oxidation of pyruvate. Or, one can start off with a system which in presence of ATP will convert acetate to acetyl CoA. In the oxidative system, phosphate is not needed for the generation of acetyl CoA. In the system involving free acetate, ATP must be supplied as energy source.

CARTER: Is there further discussion? I think Dr. Green is to be complimented upon his selection of terminology, "the sparking phenomenon." Everyone always realized that sparking was an autocatalytic phenomenon, and I think it fits in pretty well.

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PROBLEMS AND EFFECTS OF RADIATION ON CAPILLARY PERMEABILITY

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THREE FIGURES

Capillaries are the physiological section of the vascular system; they carry to the tissues substances essential for normal function of the component cells, and they remove from the tissues the waste products of cellular metabolism. Capillary permeability in its relation to radiation is therefore of importance because the effects of irradiation on tissues are imperfectly understood, and because it has long been accepted by many radiologists that some of these effects are mediated by changes in capillary permeability. In this paper, evidence for that belief will be examined and methods discussed for getting additional and more accurate information.

The anatomical structure of capillaries is complicated (Danielli, '44) in that they consist of both endothelial cells and intercellular cement substance. The latter has pores of variable size through which filtration occurs (Chambers and Zweifach, '47), but in addition water and probably electrolytes (Cowie, Flexner, and Wilde, '49) diffuse through the cells themselves. When we speak of changes in capillary permeability we should be aware that we cannot readily distinguish between changes in the cells and those in the intercellular substance. Consequently, in speaking of effects of irradiation on permeability it seems better to begin with a material so simple that we can confine our attention to the living cell itself.

RADIATION AND THE PERMEABILITY OF ISOLATED CELLS

A number of years ago, Lucké and McCutcheon measured permeability to water in the egg cells of the sea urchin, *Arbacia punctulata.* The cells are well adapted to this purpose because of their regular spherical shape, which allows volume changes to be readily measured; also because viability and injury can be tested by adding sperm, and observing whether cleavage occurs normally.

The method consists in placing cells in anisotonic solutions and measuring the volume changes as water enters or leaves the cell as the result of differences in osmotic pressure. Permeability is expressed as the volume of water passing through unit surface area per minute per atmosphere of pressure. In these experiments, permeability to water was found to be increased by such injurious agents as heat and solutions having very low osmotic pressures.

Recently, with greatly improved methods, Lucké, Ricca, and Parpart ('50) have investigated the effects of roentgen irradiation on permeability to water and to two ethylene glycols.[1] The total amount of irradiation was 100,000 r, delivered in 14 minutes. In these experiments, the cells, following irradiation, were placed in sea water made hypotonic by addition of distilled water. At once water began to enter the cells, causing their volume to increase, and such changes in average volume of many thousands of cells were measured at intervals of a few seconds by means of a diffraction method. When volumes are plotted against times, curves are obtained from which the rate of volume changes, and hence of permeability to water, may readily be computed.

The result of these experiments was that there was no difference in permeability to water between cells that had been irradiated and cells that had not. Similarly, permeability to ethylene glycol and to diethylene glycol was not affected by irradiation. When, however, sperm was added to the cell suspensions, the irradiated cells underwent cleavage tardily and atypically, and finally disintegrated; whereas, the control cells divided and developed normally. Thus it was found that irradiation had damaged one function, cell division, without altering another, permeability. Such a differential effect of injurious agents is not uncommon in biological material. Presumably, irradiation of some greater intensity would have altered cell permeability, too; but whether this would occur without disruption of the cells is uncertain.

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[1] The factors used were two tubes in alternate parallel cross-firing in center; 182 kv, 25 ma per tube, with an 0.15-mm copper equivalent filter. The target object distance was 9.5 cm, and the rate of delivery 7190 r per minute.

Similarly Reed ('48) exposed the ova of a sea urchin, *Strongylocentrotus purpuratus,* to ultraviolet light of various wave lengths (2483–3130 A) and found no significant effect on permeability to water, to ethylene glycol, or to certain other solutes.

Measurements of permeability in capillaries. Compared with these isolated cells, measurement of permeability in capillaries is complicated and subject to important errors. The methods that have been used, and the results of irradiation, if known, are given in the following paragraphs.

Direct measurement of capillary permeability to water was made by Landis ('27). A capillary in the exposed mesentery of the frog was observed with the microscope while one end of the vessel was blocked by light pressure with a glass rod. At the other end of the capillary, attention was focused on a single erythrocyte, and its rate of progress toward the obstructed end was measured, its rapidity varying with the amount of water filtered out of the capillary. Permeability was defined as the volume of fluid passing through $1\mu^2$ of capillary wall per minute under a pressure of 1 cm^3 of water. This pressure depends on the difference between hydrostatic pressure, which was measured directly by means of a micropipette inserted into the capillary, and the effective osmotic pressure of the plasma proteins, which is known.

Measured in this way, permeability to fluid was increased about 10 times by washing the mesentery with 10% alcohol in Ringer's solution, and was increased 4 times by a three-minute period of oxygen lack (Landis, '28).

This procedure is difficult and has several possible sources of error, such as changes in permeability induced by manipulation and by changes in hydrostatic pressure during the time of observation. This direct method has not been used to study the effect of irradiation.

In the second method advantage is taken of the fact that, under ordinary pressures, colloids such as certain dyes and serum proteins are unable to escape from normal capillaries, or do so very slowly; whereas, they pass readily after the capillary is injured. Thus Landis ('27) found that brilliant vital red does not escape from a frog's capillary at hydrostatic pressures under 15 cm of water, but quickly escapes and stains the surrounding tissues at all pressures when the capillary has been injured with ether.

Effect of irradiation on capillary permeability. Using the dye method in purely qualitative experiments, Graham ('43) irradiated the abdominal skin of the guinea pig by means of a carbon arc, at a distance of 1 m, for 30–40 minutes. Four hours later, trypan blue was injected in the ear vein. After two hours, the irradiated skin was more intensely blue than the unirradiated skin; the increased intensity of color was preventable by desoxycorticosterone or cortin. It was concluded that ultraviolet rays increased capillary permeability to trypan blue.

Roentgen-ray effects on capillary permeability to a dye were studied by Rigdon and Curl ('43). Numerous small patches of rabbit skin were irradiated with doses of from 680–2720 r.[2] Trypan blue was injected intravenously at various times before and after irradiation. Staining of treated skin was most intense when the dye was injected one hour after irradiation, while there was no difference from control areas in intensity of staining when the dye was injected 2–21 hours after irradiation.

Thus, while irradiation appeared to increase capillary permeability immediately, the effect was extremely transient.

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[2] The factors used were 86 kv (peak), 5 ma, 7.5 minutes, 20 cm target-skin distance, no filter. The machine delivered 272 r per minute.

Other available methods. An interesting variation of this method, making it semiquantitative, is to attach radioactive substances to the dye or other colloid. By means of a Geiger counter the relative accumulation of dye is found in different parts of the body, and, from such data, information may be obtained as to capillary permeability in different regions.

In this way Moore and Tobin ('42) studied the effect of inflammation[3] on vascular permeability, injecting Br^{82} as dibromotrypan blue intravenously in rabbits. The dose was 1.5–3 μc. One hour later, counts were made over various parts of the body. In normal animals, the greatest number of counts per minute was found over the xiphoid region, reflecting the accumulation of blood, and therefore of radioactive dye, in liver, spleen, and neighboring splanchnic regions. When the counter was moved caudad or cephalad from the xiphoid, counts fell off in a linear manner.

When inflammation was induced in some part of the body, the count went up over that region, and the normal linear distribution of counts with respect to the xiphoid was distorted in a characteristic pattern. This result was believed to indicate that more dye had escaped from capillaries in the inflammed area than elsewhere, and was taken as an indication of increased capillary permeability. No use has been made of this procedure in studying the effect of radiation on permeability.

Another semiquantitative variation was devised by Menkin ('30). He exposed the mesentery of the frog, and injected trypan blue into the heart. Under the microscope, the capillary mesentery blood became blue, and was compared in intensity with that of glass tubes containing known concentrations of the dye. With time, the color intensity in the capillaries faded, as dye escaped from them. When peritonitis had previously been induced, the rate of fading, and hence the permeability to trypan blue, was approximately twice as great as in the controls. This method also could be employed for studying the effect of radiation.

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[3] Inflammation was induced by injecting virulent staphylococci in melted agar into the subcutaneous tissues.

A carefully controlled method was devised by Benditt et al. ('50) for measuring the rate of disappearance of the colloidal dye, Evans blue, from the blood stream. The dye was injected into the tail vein of the rat, blood samples were withdrawn at intervals, and the concentration of dye determined colorimetrically. Besides changes in dye concentration, changes in permeability were judged by loss of plasma proteins and by hematocrit readings, and for these estimations blood volume determinations were necessary. In these experiments, the effect on permeability of hyaluronidase and the counter-action of cortisone were studied, but the method seems admirable for investigating the effects of radiation.

Fragility of capillaries. I will now speak briefly of the effect of irradiation on the fragility of capillaries. Fragility differs from permeability in that permeability refers to penetration through a capillary wall that is intact; whereas, fragility refers to disruption of the capillary with resulting petechial hemorrhages.

Griffith, Anthony, Pendergrass, and Perryman ('47) tested the effect of α particles on the fragility of rats' capillaries. Radon ointment [0.5–1.5 cm^3; 500 ESU (200 $\mu c/cm^3$)] was injected into the peritoneal cavity, and fragility was tested 1–8 weeks later. Under general anesthesia the peritoneal cavity was opened and the abdominal wall rolled back, exposing an artery and a vein. The abdominal wall was grasped on each side of the vessels and stretched until the vein, but not the artery, was occluded. After three minutes the tissues were released. Petechial hemorrhages, usually from 6 to 8 in number, developed in all the irradiated animals but in none of the controls. It was concluded that capillary fragility is increased by adequate irradiation.

This conclusion received support from the carefully controlled experiments of Field and Rekers ('49). Following whole-body irradiation of dogs with roentgen rays,[4] many animals died, and the survivors had numerous hemorrhages and few blood platelets; also, coagulation time was increased.

When the animals received rutin both before and after irradiation, fewer animals died, and survivors had fewer hemorrhages, though thrombocytopenia and prolonged coagulation time persisted. Rutin was thought to act directly on the vessels and to increase their resistance to the deleterious effects (fragility-increasing?) of roentgen rays. It is important to recognize that radiation causes hemorrhages not only through its effect on vascular permeability, but that thrombocytopenia and prolonged coagulation time, the latter possibly due to increased heparin in the blood (Allen and Jacobson, '47), may also be factors.

Results similar to the foregoing were reported in a recent paper by Sokoloff et al. ('50).[5]

Though it seems likely that irradiation increases capillary fragility, a definite statement cannot justifiably be made as to the effect of radiation on capillary permeability. The experiments of Graham ('43), and of Rigdon et al. ('43) suggest a transient increase in permeability that would be gone even before skin erythema would appear. What we should really like to know about is the time beginning a day or two after irradiation and covering the succeeding weeks, the time of capillary dilatation, edema thrombosis, and fibrosis, but no data on permeability during that time are available.

PLANNING FUTURE EXPERIMENTS

Surely it should not be too difficult to devise experiments that would give information, at least of a qualitative nature. I would suggest the following.

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[4] The following factors were used: 250 kvp at a target-skin distance of 37 inches. A 14.22-mm parabolic aluminum and an 0.53-mm copper filter were used. The dosage usually delivered was 350 r.

[5] Flavonoids do not always protect against irradiation damage (Cronkite, Chapman, and Chambers, '51).

1. According to Landis ('34), the most indisputable evidence of increased permeability is capillary stasis. By that term is meant that capillary flow has ceased because the plasma has escaped from the capillary and there remain only packed erythrocytes. The web of the frog's foot may be irradiated with various doses in different individuals, and the web examined with the microscope at intervals after irradiation to see if there is capillary stasis. If this occurs, in properly controlled experiments, it may be concluded that capillary permeability has been increased by irradiation.

2. In a series of various species of such mammals as mice, rats, guinea pigs, and rabbits, the abdominal skin should be irradiated. At intervals up to several weeks one of the animals should be injected intravenously with trypan blue, and the degree of staining of the irradiated skin compared with that of control areas. With larger species, such as rabbits, one ear should be irradiated, and the permeability of the cutaneous capillaries investigated. This method may be made more precise by attaching radioactive substances to the dye, as in the experiments of Moore and Tobin ('42).

If such preliminary experiments yield positive results, more elaborate and critical ones may be planned, using the quantitative methods of Landis ('27), or Benditt et al. ('50).

Errors of interpretation. I now come to certain errors that should be avoided in interpreting the results of irradiation.

1. Intercellular edema may, it is true, be caused by increased capillary permeability but, on the other hand, it may result from prolonged capillary dilatation and slow venous return (Chambers and Zweifach, '47), changes that commonly follow irradiation.

2. Capillaries are normally impermeable to a dye only if it is nondiffusible, and even this may escape at high hydrostatic pressures though permeability is normal.

3. Lastly, swelling of tissue cells, often regarded as evidence of increased cellular permeability, should be interpreted with caution, for it may mean active growth or changes

due to increased intracellular osmotic pressure resulting from injury.

SUMMARY

We may now summarize our information about the results of irradiation on capillary permeability, information which, as you have heard, is very scanty. From the few data available, it seems likely that there is an immediate but transient increase in permeability. As to capillary permeability at various periods following irradiation, data are not at hand, though it is about the later effects of irradiation that information would be most valuable. The most promising methods for obtaining this information, it seems to me, should be based on (1) the tendency for increased permeability to result in stasis of the circulation in some of the capillaries, and (2) the ability of colloidal dyes to escape into the tissues even at low hydrostatic pressures when capillary permeability is increased.

DISCUSSION

CHAIRMAN WARREN: Doctor McCutcheon's paper is open for discussion.

DOUGLAS E. SMITH: There are certain evidences, published and unpublished, which pertain to this subject. For example, Dr. Elizabeth Painter at the Metallurgical Laboratory made observations on the web of the frog's foot after X irradiation and, on the basis of separation of tissues and swelling of the web, concluded that capillary permeability was increased.

MCCUTCHEON: At what time after irradiation?

SMITH: From 1 to 14 days.

MCCUTCHEON: I am glad to know about that. I am sure that there are references that I did not find. I would appreciate your giving them to me.

SMITH: More recently some preliminary observations have been made by Dr. Schiller at the University of Illinois, using fluorescein and fluorescein-labeled proteins.

MCCUTCHEON: That is a diffusible dye, is it not?

SMITH: Yes. But the fact that he labeled plasma proteins with it removes this objection. He has made some observations on the ultraviolet-irradiated skin of the rabbit, and has found indications of increased permeability.

At Argonne we are working with bat-wing preparation and have found indications of increased permeability after X irradiation. Our technique consists in irradiating a local area, say a centimeter in diameter, and then injecting radioactive phosphorus into the animal at various times after irradiation. The animal is sacrificed, the wing removed, and radioautographs made of the irradiated and nonirradiated regions. Invariably there are greater concentrations of radioactive material in the irradiated portions. In these same areas there is no dilatation of blood vessels and no hemorrhage.

McCUTCHEON: That is a rather unique advantage of that material, is it not?

SMITH: Yes. It so happens that, in the bat, either with local irradiation or with whole body irradiation (up to about three weeks, the extent of our observations), we never saw evidences of changed vessel diameter or hemorrhage even after very high radiation dosages.

PATT: There is another piece of work by Dr. Painter that is pertinent here. Doctor Painter repeated Rigdon and Curl's experiments, using Evans blue. Moreover, she also followed the appearance of the dye during the period of late erythema in the rabbit, about 10 days to 2 weeks after irradiation. She found, as did Rigdon and Curl, an increased appearance of Evans blue immediately after irradiation. By 10 days after irradiation, however, there was no difference in the appearance of Evans blue between the irradiated and nonirradiated areas. Yet, interestingly enough, when fluorescein was injected 10 days after irradiation it appeared more rapidly in the irradiated than in the nonirradiated area.

HARRINGTON: In living preparations of grasshopper embryos a swelling of the neuroblast cells occurs after doses of X radiation as low as 100 r. This increase in cell size occurs almost immediately, and therefore cannot be attributed to growth of the cells. Measurements of nuclei diameters in fixed preparations reveal a statistically significant difference in size of nonirradiated and irradiated nuclei; the latter have a greater volume. This change in volume has been observed at least 10 hours after irradiation. The depolymerization or molecular rearrangements of desoxyribonucleic acid, as indicated by a spectrophotometrically determined decrease in stainability of nuclei with methyl green after X irradiation, may contribute to an increase in osmotic pressure which causes the cells (and nuclei) to swell.

FURTH: I believe Dr. Flexner said that, at a certain stage of scientific development, certain experiments and certain discoveries become inevitable. Many of those things that Dr. McCutcheon has suggested were done at the Oak Ridge National Laboratory; and several others are in the immediate plan or are being done. Very little of this has appeared in the literature, only one in the Proceedings of the Society of Experimental Biology and Medicine, and others in our quarterly reports. I shall give you some of our studies on

ENDOTHELIAL FRAGILITY BY IONIZING RADIATION[6]

The studies I should like to describe concern changes in permeability of the endothelial membrane following irradiation; these changes may not be on cellular but on intercellular level.

We began our work, with several members of the Biology Division, on the effects of X rays on macrophage function. This was tested with colloidal gold (with Dr. Sheppard), labeled heterologous and homologous plasma, and erythrocytes. Whichever of these substances was introduced into the blood stream it was found to disappear faster from the blood of irradiated animals than from that of normal controls (Wish et al., in press). A material balance of the in-

[6] The work described by Dr. Jacob Furth, Biology Division, Oak Ridge National Laboratory was performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

jected colloidal radiogold or I^{131}-labeled protein can be made with ease. First, the radioactivity of the entire body is measured with the aid of a high-ionization γ chamber and then the activity of each organ is tested separately at various times after injections of the tagged material. Radioautography aids in further localization of the tagged substances. The essential results of these experiments to be sketched were found in mice, rats, rabbits, swine, dogs, and man.

Colloidal gold proved to be useful in assaying phagocytic function which was found not to be markedly altered after irradiation. A material balance of injected I^{131}-labeled plasma made after mixing time (5–10 minutes) led to a simple technique for determining organ plasma volume (Wish et al., '50). The puzzling question was, however, where did the various substances go after disappearance from the blood of the irradiated hosts during the first hour after injection. We were particularly puzzled that even auto-erythrocytes (erythrocytes taken out of the blood of an animal, tagged with P^{32}, and reintroduced) disappeared faster and in larger quantities from the blood of X-rayed animals than from that of normal controls.

It became necessary to test the other end of the stream, i.e., the lymph. Permanent lymph-venous anastomoses were prepared in dogs as described by Brown and Hardenberg ('50; Bigelow et al., '51). One plastic cannula was inserted in the thoracic duct, another into a vein, and the connecting loop was exteriorized. The dogs so prepared live comfortably and their lymph can be studied after irradiation for one to two weeks. The loop can be easily disconnected, the lymph and blood sampled, and thus the rate of clearance of substances introduced into the blood studied with ease (Bigelow et al., '51; Ross et al., '52). In normal dogs the loops usually became closed within a few days. In X-rayed animals, the loop remained patent because of the prolonged clotting time, and the animal could be studied for as long as 16 days. In one dog three successive clearance tests could be made with I^{131}-tagged homologous plasma and thiocyanate at 3- to 4-day intervals (Storey et al., unpublished data). In the rat, the lymph was drained by the technique described by Bollman et al. ('48).

The presence of a large number of red blood corpuscles in the lymph was the most striking finding after massive irradiation. About 6 days after irradiation of dogs the number of erythrocytes in the lymph begins to rise and after 7–14 days there is a sudden spurt, the red blood count in lymph rising up to 1,000,000 so that the lymph becomes grossly bloody. Similar findings were obtained in rats. Figure 1 (from Ross et al., '52) is given as an example.

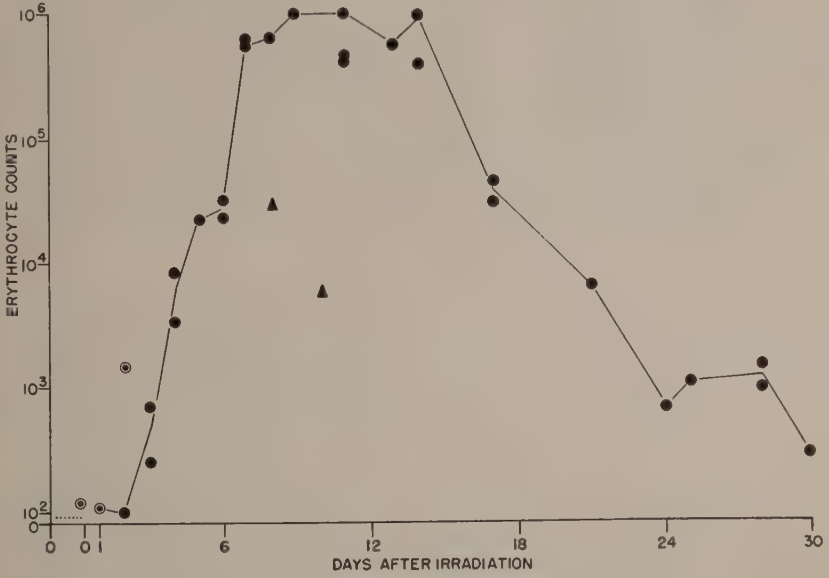

Fig. 1 Erythrocyte counts in the lymph of rats exposed to 650–750 r of X rays. Each point is obtained on a different animal.

At autopsy of massively irradiated animals dying during the hemorrhagic state, the thoracic duct was as red as a vein, as were many lymphatics. The report of Brown et al. ('50) is not contradictory since their study of the animals was made during only the first week after irradiation. The hemorrhagic phenomena are "delayed" and in dogs they become distinct only during about the second week after irradiation. Red blood cells may appear earlier in the lymph, but a marked rise usually begins abruptly during the second week. The number of erythrocytes in the lymph of X-rayed animals is increased even when there is no gross evidence of hemorrhage.

A characteristic and long-known finding after massive irradiation is the hemosiderosis of the lymph nodes (Dunlap, '42; Tullis, '49). General de Coursey ('48) has noted this in people exposed to atomic explosion. The meaning of this change has hitherto not been fully appreciated. When blood cells are destroyed in the great circulation, they are filtered out in the spleen and the hemoglobin is converted into hemosiderin by macrophages (reticuloendothelial cells) of the liver and spleen while similar changes in the marginal sinuses of the lymph nodes may indicate extravasation and destruction of erythrocytes in tissue spaces (fig. 2 A, B, C, D). This phenomenon, thus far missed, is present to a variable degree in almost every massively irradiated animal, even in species in which death is not attributed to hemorrhagic manifestations. E. Clark noted in the transparent chamber of rabbit ears that, when erythrocytes escape in tissue spaces, they disintegrate or are removed by macrophages (cf. Ross et al., '52). The gross appearance of lymph nodes and lymphatics indicates that this change occurs after irradiation throughout the body.

The pathogenesis of massive anemia after irradiation has never been satisfactorily explained because erythrocytes are very resistant to irradiation *in vitro*. The above observations may serve to explain the erythrocyte destruction in irradiated hosts. Experiments were performed in association with Dr. Kahn to study this process more closely (Kahn and Furth, '52). Fe^{59}-labeled erythrocytes were introduced in two sets of rabbits, one irradiated immediately before, the other immediately after injection of labeled erythrocytes. Thus, the hosts of both sets of animals received the same dose of X rays, but in only one set were the erythrocytes irradiated.

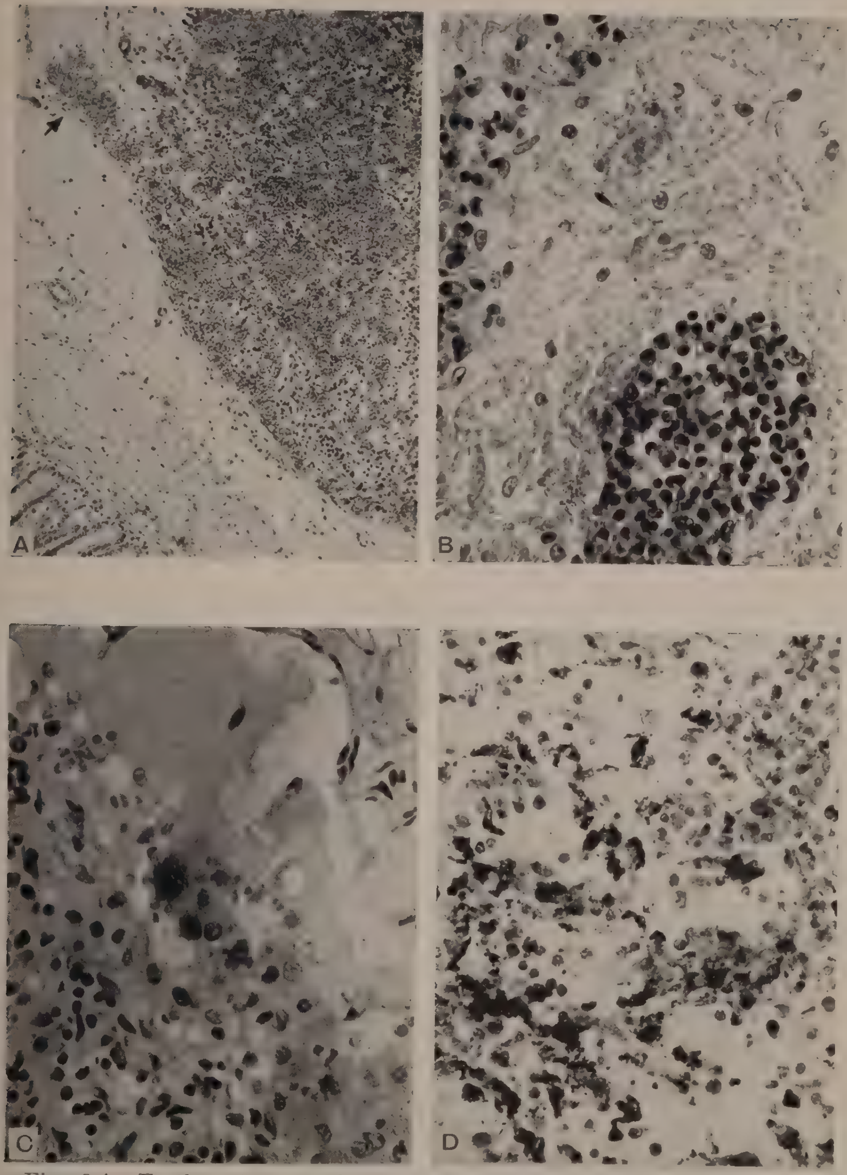

Fig. 2 A Erythrocyte mass in the mesenteric lymph node of a massively irradiated rat. The arrow points to an afferent lymphatic filled with blood.

B The sinuses of an irradiated dog filled with erythrocytes. The nucleated cells are predominantly plasma cells which characteristically replace the normal lymphocytes.

C Massive edema fluid distends a lymph sinus of an irradiated rabbit.

D Hemosiderosis of the lymph node (macrophages filled with black granules) of an irradiated rabbit. The erythrocytes are no longer present in the expanded sinuses.

The changes found in the erythrocyte mass and plasma volume were alike in these two sets of animals. Thus the major change affects, primarily, the hosts and not the erythrocytes. The Fe^{59} activity drops in irradiated animals, notably in those fatally X-rayed, beyond that attributable to death of aged erythrocytes. A small amount of radioiron is lost from the body after irradiation. The radioactivity of the lymph nodes and spleen per gram of organ becomes greatly increased in X-rayed hosts due to massive hemosiderin deposits in these organs (Kahn and Furth, '52). The latter is measurable and gives an approximate quantitation of the total number of erythrocytes destroyed.

Thus diversion of erythrocytes into lymph and tissue spaces is a major factor in the causation of anemia, other factors being (1) diminished production due to bone marrow depression and (2) erythrocyte destruction. The latter may be related to passage of erythrocytes through tissue spaces or to an unknown hemolytic factor.

The pathogenesis of the capillary fragility is unknown. An indirect damage seems to us to be more likely than a direct radiation injury. Very recently Cronkite and associates ('51) attribute the hemorrhagic phenomena of irradiation to lack of platelets. If this proves correct, the hypothesis may be advanced that platelets are normally needed for the integrity of the intercellular capillary cement, to the formation of which they steadily contribute under normal (physiological) conditions. There are other possibilities likewise amenable to experimental analysis. Whatever the pathogenesis of the capillary fragility it appears to be a self-aggravating process as a sudden diversion of erythrocytes into lymph spaces causes anemia and anoxia and the latter increases the capillary permeability.

The degree of permeability is being measured in fistula dogs by determining the concentration of intravenously injected I^{131}-labeled albumin in the blood and the lymph at various intervals after the injection. The labeled plasma enters the lymph very slowly and equilibrium is approximated after about 6–10 hours. After this time the radioactivity in blood and lymph (indicating albumin concentration) drops in about parallel fashion. This drop indicates the rate of metabolism of the injected albumin (fig. 3). The amount of loss of albumin from the blood when equilibrium is reached minus the amount metabolized indicates the quantity of albumin diverted into the lymph; this albumin mass is related to the lymph volume. These observations led to a technique of approximating the lymph space (Storey et al., '51). Similar studies have been conducted in normal dogs by Wasserman and Mayerson ('51) who arrived at essentially the same conclusions and problems as concern the normal animal. Knowledge of the average ratio of albumin concentration between blood and lymph is needed for the exact determination of the lymph volume. However, the lymph is a heterogeneous fluid, different in different lymphatics and tissue spaces. Thus, taking the thoracic duct as an average lymph, the lymph volume can be approximated only crudely. The determination of the metabolism rate during the equilibration time can likewise be only approximated, this rate being variable and having a tendency to drop. The following formula devised by

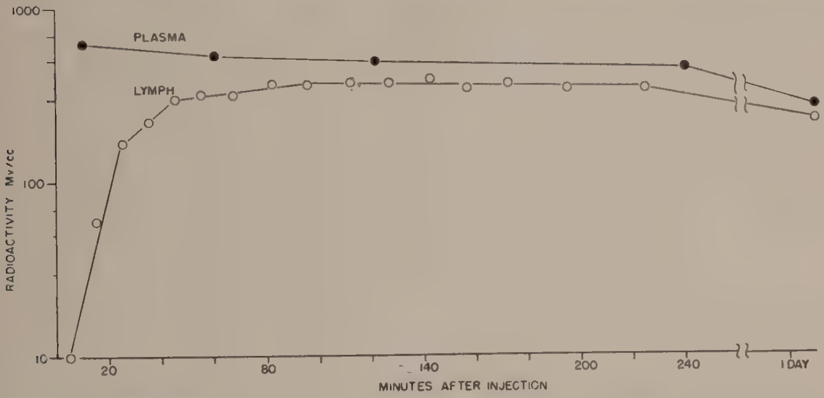

Fig. 3 Radioactivity/cm^3 vs. time after injection of plasma and lymph of a normal dog injected with I^{131}-labeled canine albumin.

our consulting mathematicians may be used to estimate the lymph volume (Storey et al., '51).

$$R^{*-kt} = V_p \; R_p^{(t)} + V_l \; R_l^{(t)}$$

where

R^* = total radioactivity injected
k = exponential rate of albumin metabolism
t = time
V_p = volume of plasma
$R_p^{(t)}$ = radioactivity per milliliter of plasma measured at time t
V_l = volume (space) of the lymph
$R_l^{(t)}$ = radioactivity per milliliter of lymph measured at time t.

The calculation of lymph volume requires knowledge of the total quantity of albumin lost from blood during the equilibration time, ratio of albumin concentration between blood and lymph, and metabolism rate (k value). A slight change in the latter greatly alters the calculated lymph volume. Thus a method for the exact determination of the lymph volume remains to be worked out, but it is possible to compare the various values obtained in parallel studies between X-rayed and normal dogs. These indicate the following changes in X-rayed hosts: (1) an increased rate of entry of intravenously injected albumin into the lymph, (2) a frequent increase of albumin concentration in the lymph, and (3) a variable and frequently increased estimated lymph volume. The metabolism rate of injected labeled albumin was found to be essentially unchanged.

A VOICE: How long after irradiation do you find those changes?

FURTH: Increase in permeability is not evident within 24 hours after irradiation. If we take the presence of erythrocytes as an indication of capillary fragility, there is in dogs little damage during about the first 6 days; later the number of red cells in lymph rises suddenly. At the 13th or 14th day there are about 1,000,000 red cells per cubic millimeter in the lymph of both dogs and rats. A crisis seems to occur during the hemorrhagic period, after which the animal either dies with profound anemia or slowly recovers.

VICTOR: These various methods that are being proposed for measuring capillary permeability have much the same principle; and in addition to some of the errors that have been pointed out, there is one possible source of error that I think has not been mentioned.

For example, some of the experiments that Dr. Furth showed us indicate a definite relationship between the concentration of protein in the lymph and that in the blood stream, a relationship which is evident from the distribution of dye, as was shown by Lamson almost 30 years ago, and which is now demonstrated with radioactive proteins. The same relationship, I think, applies to both types of experiments.

On the other hand, there is a source of error in analyzing or interpreting alterations in these relationships as changes in permeability. A little over 20 years ago some experiments showed that the rate of disappearance of brilliant vital red from the blood decreased for about 3–4 weeks after injection of India ink (Victor et al., '30). It was shown that this effect was not due to the carbon particles, but to something else in the ink. There was some functional, nonmorphological change in the liver because, when the rate of elimination of the dye was studied in the bile, it was found that the dye was retained. Therefore, alteration of dye excretion would affect its rate of disappearance from the blood stream.

What are other conditions that might lead to dye retention? If one injects trypan blue into an animal, the dye follows the same pattern as brilliant vital red in its disappearance from the circulation. The dye can be found in the blood as long as 5 days later. But all this time the dye is being concentrated in cells throughout the body, mainly in histiocytes and in epithelium of the renal proximal convoluted tubules.

Now, if one takes the experiments of Moore and Tobin, in which radiation lesions contain histiocytes and fibroblasts as well as dilated capillaries, the increase in concentration of test material might represent only what has been deposited in those histiocytes, and that would be no expression whatever of a change in capillary permeability.

I think, therefore, instead of using only beautifully simple techniques for measuring radioactivity or density of dye, it must be established that one is measuring a well-defined factor that is leading to the deposit of the dye or the radioactive material.

von Sallmann: It has been shown repeatedly that capillary systems of various organs in various animals differ in their permeability for dyes and serum proteins. In reference to Dr. McCutcheon's observations on capillary systems other than those of the eye, this fact was demonstrated in experiments which we carried out a few years ago on the blood aqueous barrier of rabbits. The barrier is located in the ciliary processes and consists, in its most important part, of an extremely dense network of wide capillaries. The permeability of the barrier for fluorescein was examined *in vivo* on the undisturbed globe. Appearance and disappearance curves of the dye in the aqueous humor after intravenous injection could be compared in the irradiated eye with the curves in the nonirradiated control eye of the individual animals.

The permeability for the dye was increased over a period of 24 hours when the eye had been exposed to a dose of 250 r of penetrating X rays. Irradiation with 500 and 1000 r augmented the entry of the dye into the aqueous humor from the blood stream for a period of one week. When a dose of 2500 r was used, the permeability of the barrier was found increased over a period of 5 weeks. With this dose of X rays the entry of the diazo dye T1824 from the blood into tissue interstices of the anterior portion of the uvea exceeded that in the control eye considerably, and the values of the serum proteins in the aqueous humor showed nephelometrically a level about 20 times higher than the levels in the control eyes. Attention should be drawn to the fact that observations on the permeability of the ciliary capillaries for fluorescein can be carried out under physiological conditions *in vivo* without any irritation of the eye by dissecting procedures or withdrawal of the test fluid.

WARREN: Thank you, Dr. von Sallmann. Doctor McCutcheon hinted at one point in the opening portion of his talk on which I would like to see if he has any further evidence — this question of the passage of particulate material through intercellular cement substance as well as through cells. Are there any data as to whether or not the particulate material, such as the large molecule dyes, passes through cells as it is picked up, let us say, in the endothelial cells; or whether it is actually passing through these pores in the intercellular cement substance?

MCCUTCHEON: I am sure that Chambers and Zweifach would say that the particles pass through the intercellular cement substance. I believe that is the general opinion, according to existing data.

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NERVE FUNCTION AND IRRADIATION EFFECTS

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ELEVEN FIGURES

The rapid development of nuclear physics with the resulting possibility of the use of atomic energy will inevitably and profoundly affect the life of our society. In war and in peace we will be increasingly exposed to irradiation effects. In addition to the theoretical interest of how radiation acts upon biological systems, the practical problems will force medicine to an intensive research of this question.

Information about the effects of irradiation has been slowly accumulating ever since treatment with X rays has been applied to tumors. However, since the development of atomic energy, with its obvious dangers and potentialities, the study of the biological effects of ionizing radiations has been greatly increased. Many investigations were devoted to effects on the whole animal, whereas, little information is available on the finer mechanisms. In only a few instances the radiation effects have been studied on molecular level, as for instance, the inactivation of sulfhydryl groups by ionization of water and formation of oxidizing groups, demonstrated by Barron and his associates (Barron et al., '49; Barron and Dickman, '49; Barron and Flood, '50). Obviously, a satisfactory explanation of the effects of atomic energy on biological systems will eventually require analysis at a molecular level.

Since my assignment is to present the problems of nerve function, I would like first to discuss present concepts of the elementary process of the conduction of the nerve impulse, which must form the basis of any investigation of this problem. Then I will outline some possible ways of approach which appear to me to be valuable in the study of irradiation effects.

PHYSICAL EVENTS DURING NERVE ACTIVITY

The so-called membrane theory, developed at the end of the last century, is still the basis of all modern concepts of conduction, although slight modifications became necessary. The theory assumes that the nerve axon is surrounded by a polarized membrane with the positive charge on the outside and the negative on the inside. During activity the charge is reversed at the point where the impulse passes. This was demonstrated in experiments with the giant axon of squid, in which electrodes were inserted into the interior of the axon and the changes of the membrane potential measured directly across the membrane (Curtis and Cole, '42; Hodgkin and Huxley, '45). Flow of current is generated from the resting to the active region whereby the adjacent region is stimulated. There the same process is repeated. In this way successive parts of the surface are stimulated and the nerve impulse is propagated.

It is generally accepted today that ionic concentration gradients are the primary source of the electromotive force of the action potential, as was suspected by DuBois-Reymond. As a result of the work of various laboratories, it is now well established that there exists a considerable concentration gradient of sodium and potassium ions on the two sides of the membrane: the concentration of sodium is high in the outside fluid as compared with the concentration of this ion species inside; the reverse is true for potassium ions. After the war the availability of radioactive ions made it possible to study the ion movements in rest and during activity. Such studies were carried out simultaneously and independently in our laboratory and by a group in Cambridge, England (Rothenberg and Feld, '48; Rothenberg, '50; Hodgkin and Huxley, '50; Keynes, '48, '49; Keynes and Lewis, '50). There is a constant movement of ions across the membrane even in resting condition. The equilibrium is dynamic. During activity, there occurs a rapid influx of sodium and an equivalent amount of potassium leaks to the outside. According to our data, the amount of sodium which enters per square centimeter surface per impulse is 4.5×10^{-12} mole. The most recent data of the Cambridge group are in close agreement with these figures.

It was already shown by Overton in 1902, and later confirmed by Kato ('36) and Erlanger and Blair ('38) that nerves become inexcitable in the absence of sodium ions. Hodgkin and his associates recently investigated the role of sodium ions during activity and found that the spike height is determined by the concentration of sodium ions (Hodgkin and Katz, '49a); the higher the sodium concentration, the higher the spike. The observations suggest that the entrance of sodium into the axon occurs during the ascending phase of the action potential, whereas, potassium leaks during the descending phase. The results support the assumption that, at rest, the permeability of the membrane is high for potassium ions, much higher than that for sodium ions. During activity, however, the membrane becomes more permeable to sodium ions, about 500 times more than in rest, according to the estimates of the Cambridge group.

The investigations described support the view that the ionic concentration gradients are the source of the electromotive force of the action potential in nerve and muscle fibers. This raises the fundamental question: By which mechanism does the potential source, inactive in rest, become suddenly effective? The most likely assumption is that of a trigger mechanism by which the protein (or lipoprotein) membrane becomes suddenly more permeable so that the ionic concentration gradient may become an effective force. Something must happen at successive points of the protein (or lipoprotein) membrane which opens and closes the barrier for the flow of ions. The change of the protein membrane must be produced by a quickly reversible chemical reaction. As was already postulated by Kurt H. Meyer ('37, '51), it may be assumed that the membrane is formed by long protein chains which will have basic and acidic groups. These chains may have acidic or basic character depending on the ratio of the groups. Acid proteins will be permeable to cations; the basic ones, to anions. Meyer arrived at the definite conclusion that permeability changes can be excluded as a primary source of the action currents. They must be produced by a chemical reaction. He demonstrated that ions may produce permeability changes in protein membranes of the order of magnitude observed during activity. He postulated as the primary event in conduction the release of a cation which produces the permeability changes. Meyer mentioned acetylcholine, among other possible compounds, although at that time the facts prerequisite for this assumption were not yet known.

A change in membrane resistance was experimentally established by observations on the giant axon of squid (Cole and Curtis, '39). On the basis of measurements of impedance changes they calculated that, during the action potential, the resistance of the membrane decreased from about 1000 ohms to 40 per square centimeter. Moreover, the temperature coefficient (Q_{10}) of both the rising and falling phases of the spike was recently determined and found to be high: 2 for the first, and 3.2 for the second phase (Hodgkin and Katz, '49b). This further supports the assumption that the spike is associated with chemical reactions.

CHEMICAL APPROACH TO THE STUDY OF NERVE FUNCTION

In the study of cellular mechanisms there have been only few instances of such a discrepancy between the rapid progress achieved by physical methods and the lack of information of the underlying chemical reactions as in the case of conduction. The difficulties encountered are readily understood if we recognize two outstanding features of conduction revealed by physical measurements: (a) the high speed of the process and (b) the small amount of energy required. The elementary process of conduction takes place within a fraction of a millisecond; the initial heat per gram of nerve per impulse is of the order of magnitude of 10^{-7} to 10^{-8} g calorie. No methods are available for direct analysis of compounds metabolized with this speed and in such small amounts. However, the spectacular progress of physicochemistry, protein chemistry, and especially enzyme chemistry during the last three decades made it possible to analyze many intermediate reactions, to correlate metabolism and function in various fields, and to advance the analysis of function to the molecular level. A classical example of this development is the progress achieved in our knowledge of the elementary process of muscular contraction.

Two compounds were specifically associated with nerve function during the last two decades — adrenalin and acetylcholine. It was assumed for some time that these compounds are liberated from nerve endings, that they cross a nonconducting gap, and act upon the effector organ as mediators of the nerve impulse (neurohumoral or chemical transmission). Adrenalin was believed to be the mediator at sympathetic, acetylcholine at parasympathetic nerve endings. Whereas, in the case of the autonomic system, these ideas seemed acceptable to physiologists, the situation changed when the same hypothesis was applied to the neuromuscular junction and to ganglionic synapses. Neurophysiologists vigorously objected to this view. The excitable properties of the cell body do not differ fundamentally from those of the axon (Fulton, '38, '43, '49). At a Symposium on the Synapse in 1939, Erlanger ('39) scrutinized all the facts upon which the notion of the peculiarity of the synapse was based. His data and his analysis show that the electrical signs of nerve activity do not justify the assumption that conduction along axons and transmission across synapses are basically different.

The hypothesis of chemical mediation was essentially based on two observations: (a) the high sensitivity of certain synaptic junctions to extremely small amounts of acetylcholine, and (b) the appearance of the ester in the perfusion fluid of these junctions following stimulation. The first observation is of great pharmacodynamic interest; the second shows that the release of acetylcholine is associated with nerve activity. Neither of these two observations give any indication of the precise function of the ester. The hypothesis of its mediator role was in contradiction to the conclusion based upon electrical signs. In this impasse a new approach became necessary by which a satisfactory interpretation of the observations with acetylcholine had to be found and integrated with the concepts supported by physical recordings. For an understanding of the elementary process of conduction and the role of acetylcholine in the sequence of reactions associated with this process an analysis with methods of physical chemistry and especially enzyme chemistry appeared, in this situation, imperative.

Studies of the enzymes which hydrolyze and synthesize acetylcholine have revealed many significant facts pertinent for the evaluation of the role of the ester in nerve function. It was, moreover, possible to correlate the activity of these enzymes with the electrical manifestations of conduction, to establish the sequence of the chemical reactions and energy transformations responsible for the generation of the electric currents, and to integrate appearance and disappearance of acetylcholine into the metabolic cycle of the nerve cell. From these investigations emerged an entirely new picture of the role of acetylcholine in nerve function. It became apparent that the hypothesis of ''neurohumoral'' or ''chemical mediation'' must be abandoned. This hypothesis is today of only historical interest. By a great variety of facts accumulated during the last 15 years, it has been conclusively established that the release and removal of acetylcholine are intracellular events occurring in the conducting membrane during the passage of the impulse. The chemical reactions are essential for the generation of the electric current, but the propagating agent is the electric current. In the cyclic process generating the electric potential, acetylcholine is an essential link. As will be discussed later, the release of the ester and its action upon a ''receptor protein'' is apparently the primary event opening the barrier which keeps up the ionic concentration gradient. Hereby the potential energy source of the electromotive force becomes effective.

The information available about the enzymatic hydrolysis and synthesis of acetylcholine and about the role of the ester in nerve conduction has been reviewed (Nachmansohn, '48, '50a, b; '52; Nachmansohn and Wilson, '51). A few examples only will be presented to illustrate the approach and to summarize briefly the available information before discussing the problem of irradiation effects on nerve cells.

ESSENTIALITY OF ACETYLCHOLINESTERASE IN CONDUCTION

Conductive tissue contains a special type of esterase, called acetylcholinesterase (Augustinsson and Nachmansohn, '49; Augustinsson, '49), which has a relatively high specificity for acetylcholine and is distinctly different from other types of esterases occurring in the body. Among the typical features may be mentioned a well-defined substrate concentration optimum. The activity-substrate concentration relationship is characterized by a ''bell-shaped'' curve (Haldane) (fig. 1). Propionylcholine is hydrolyzed at a lower rate than acetylcholine; butyrylcholine, not at all at the same enzyme concentration (Nachmansohn and Rothenberg, '45). Noncholine esters are split at a very low rate at a substrate concentration optimum for acetylcholine; only at a very high concentration the rate of hydrolysis increases. The affinity of acetylcholine for the enzyme is high (i.e., the Michaelis constant is small), that of noncholine esters is low. In contrast, with other types of esterase, e.g., with serum esterase, we find the usual activity-substrate concentration relationship: the rate of hydrolysis increases with the concentration of the substrate in an S-shaped curve (fig. 2); moreover, the rate of hydrolysis increases from two to 4 carbons with the increasing length of the acyl chain. Other types of esterases differ still more in many respects.

Acetylcholinesterase is present in conductive tissues throughout the whole animal kingdom. It has been found in all types of nerves, invertebrate and vertebrate, motor and sensory, central and peripheral, so-called cholinergic and adrenergic, and in muscle (Bullock et al., '47). The outstanding physiological feature of the enzyme is the high speed at which it may hydrolyze acetylcholine and thus transform the active compound in its two inactive parts. This process may occur in a few microseconds. On the basis of electrical signs it must be postulated that the chemical reaction associated directly with electric manifestations should occur within less than a hundred microseconds. The speed of the activity of acetylcholinesterase satisfies this postulate (Rothenberg and Nachmansohn, '47).

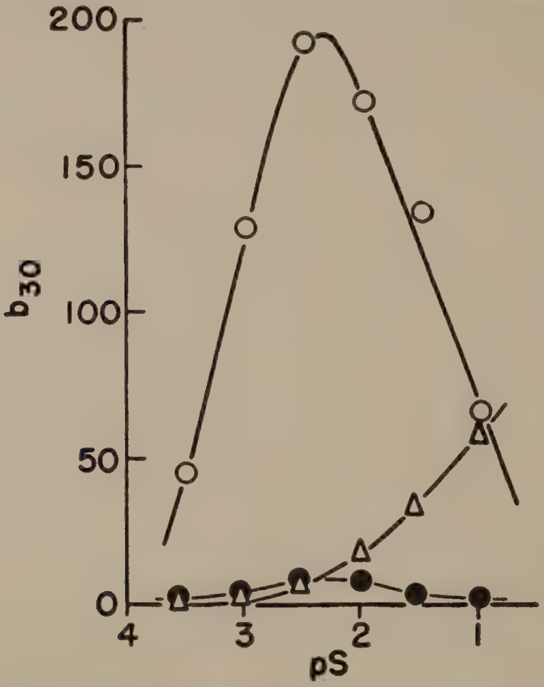

Fig. 1 Activity — pS curves for the enzymic hydrolysis of some esters by acetylcholinesterase (extract from nucleus caudatus of ox). ACh (○) = acetylcholine, BuCh (●) = butyrylcholine, TA (△) = triacetin (Augustinsson, '49).

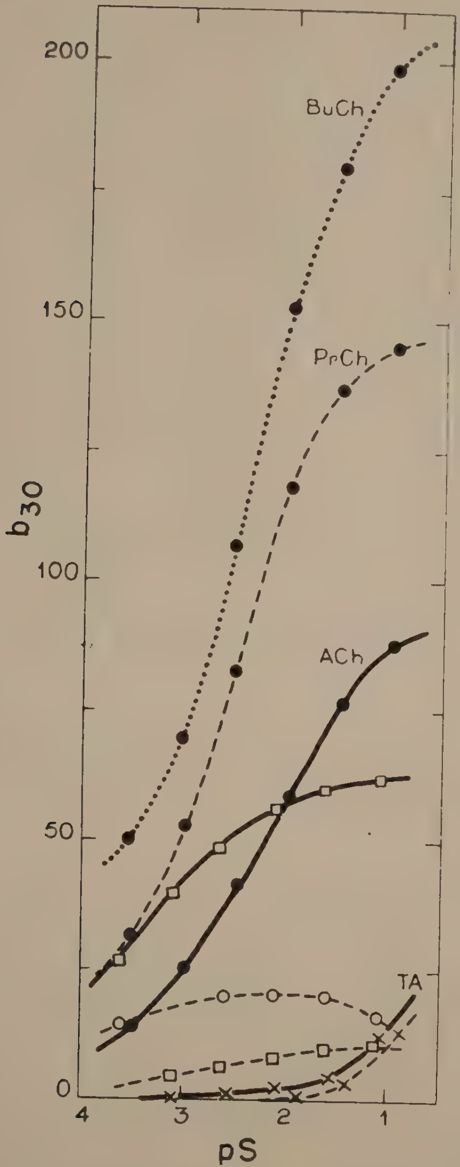

Fig. 2 Activity — pS curves for the enzymic hydrolysis of some esters by serum esterase (pumped preparation from human blood serum).

The concentration of the enzyme is high in all nerve tissue. Significant amounts of ester may be split within a millisecond, i.e., within a period of time during which the impulse passes. The real significance of the high concentration becomes still more apparent in connection with the peculiar localization of the enzyme: it is localized exclusively in the surface of nerve and muscle fibers. The most clear-cut evidence has been provided by experiments with the giant axon of squid (Boell and Nachmansohn, '40). No activity was detectable in the interior of the axon, the axoplasm; all the enzyme was localized in the sheath. Since bioelectric potentials are generally assumed to be surface phenomena the exclusive localization of acetylcholinesterase in the surface is suggestive, especially in connection with other facts to be discussed later.

During the last few years considerable progress has been achieved in the understanding of the mechanism of the reactions catalyzed by acetylcholinesterase (Wilson and Bergmann, '50a, b; Wilson et al., '50; Bergmann et al., '50a, b). The progress became possible by the study of the kinetics with various substrates and inhibitors as a function of pH. The great number of different types of inhibitors and substrates available were extremely useful for carrying out these investigations. The observations have thrown new light on some problems of physiological importance and may be mentioned briefly.

The active surface of the enzyme protein has two functionally and spatially separated sites: an anionic site which attracts the cationic nitrogen by Coulombic and Van der Waals forces; and an esteratic site which forms a covalent bond with the carbon of the carbonyl group of the ester (fig. 3). Whereas, the esteratic site is probably similar in most types of esterases, the special features of the acetylcholinesterase apparently depend essentially on the anionic site. Eserine is a much stronger inhibitor at acid pH, where it is cationic, than at the alkaline range; there it is a weak inhibitor, being a base and therefore not attracted to the anionic site. Prostigmine, on the other hand, being a methylated quaternary ammonium salt like acetylcholine itself and therefore always cationic, does not show any pH dependence between pH 6 and 10 (fig. 4).

Evidence has been obtained for the existence of basic and acidic groups in the esteratic site. The basic or nucleophilic group attracts the electrophilic carbon of the carbonyl group of the ester. The electrophilic character of this carbon is a determining factor in the reaction mechanism. This has been demonstrated by substitution experiments in which the inhibitory structure of a compound increased with the increasing electrophilic character of the carbon. The dissociation constant of the basic group is about 7.0 which differs considerably from that of most amino groups. Ring nitrogen atoms in imidazol derivatives have active constants close to the value obtained and it is possible that the basic group is an imidazol derivative. Wilson (Wilson et al., '50) has proposed a mechanism of hydrolysis in which an acylated enzyme is the intermediate product. This form is highly reactive and amenable to attack by nucleophilic reagents such as water, hydroxylamine, and choline. It is pictured as an enolate ion, but this would be only one of the resonance structures. A

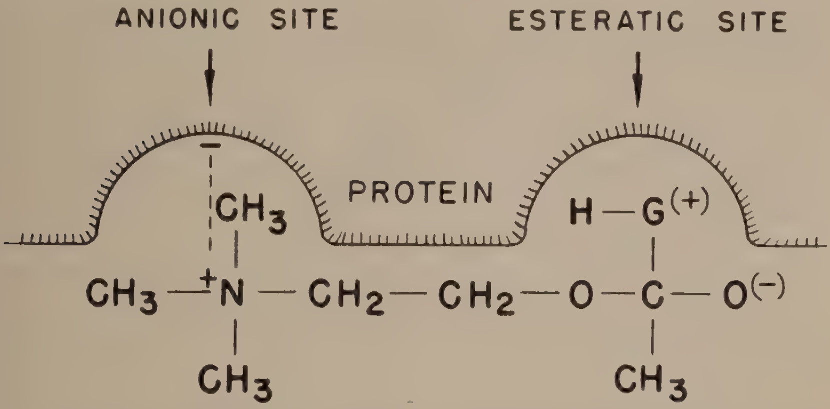

Fig. 3 Schematic picture of interaction between the active groups of the surface of acetylcholinesterase and its substrate. H symbolizes the acidic; G, the basic group.

great number of reactions have been predicted on the basis of this scheme and experimentally verified.

A frequent method of testing the significance of a chemical reaction in a cellular function is a block of this reaction by specific inhibitors. A classical example is Lundsgaard's famous observation that, after blocking lactic acid formation by monoiodoacetic acid, muscular contraction still may continue as long as phosphocreatine breakdown goes on. If the release and removal of acetylcholine are essential in the elementary process of conduction, inhibition of acetylcholinesterase should stop this function. It was demonstrated, in 1945, that inhibitors of acetylcholinesterase block conduction when applied to the giant axon of squid (Bullock et al., '46). The compounds used were reversible inhibitors; correspondingly, the effect on conduction could be reversed.

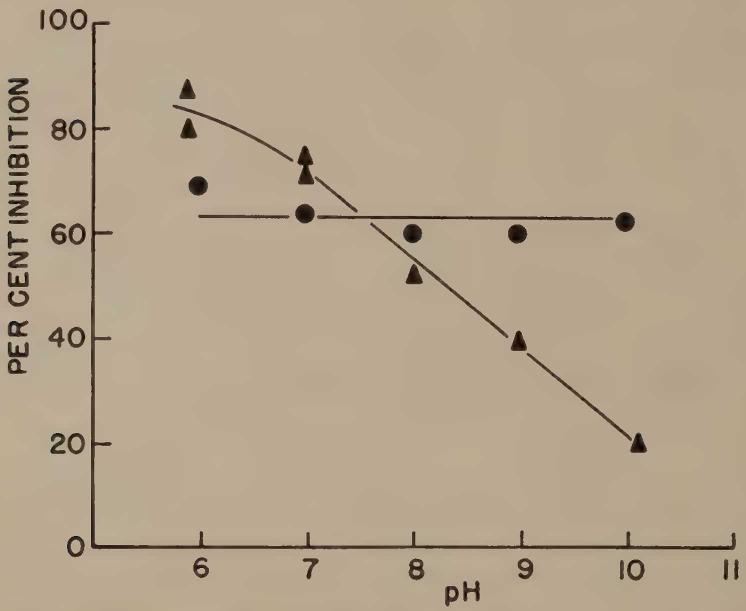

Fig. 4 Inhibition of acetylcholinesterase by prostigmine (●) and eserine (▲) as a function of pH (Wilson and Bergmann, '50a).

The discovery of a group of compounds, the alkylphosphates, which inactivate acetylcholinesterase irreversibly, initiated a new and dramatic development. Observations were described, in 1946, which seemed to contradict the assumption of the necessity of acetylcholinesterase in conduction (Crescitelli et al., '46). It was thought possible to inactivate acetylcholinesterase completely at a period when the nerve was still able to conduct. Further developments, however, changed the picture (Nachmansohn, '48). A few important results may be briefly mentioned.

The most widely used compound for the studies testing the functions of the enzyme in conduction was diisopropylfluorophosphate (DFP). The inhibition of acetylcholinesterase by this compound is a slowly progressive process. A striking parallelism between the progressive rate of irreversible inhibition of enzyme activity and the progressive rate of irreversible abolition of conduction has been demonstrated in nerves exposed to DFP (Bullock et al., '46).

Under no condition has it been possible to dissociate conduction from acetylcholinesterase activity. In some earlier investigations the fact was overlooked that only a relatively small fraction of the enzyme is necessary for intact function. In the frog sciatic nerve, for instance, this fraction is smaller than 10%. The presence of an excess of enzyme is not surprising; it is in full agreement with all experience with other enzyme systems and in fact with all physiological mechanisms. The difficulty encountered in the present case is readily recognized if we recall the fact that the chemical reaction associated with the primary event in conduction must be extremely small on the basis of the initial heat. Only an extremely small amount of acetylcholine may be required for the primary reaction, and the amount of enzyme found at the critical period when conduction begins to be impaired may consequently be very small.

The presence of excess of acetylcholinesterase has recently been demonstrated *in vivo* by de Roeth ('51). About 15% of the initial concentration was found to be the minimum necessary to maintain normal pupillary function. This minimum requirement found *in vivo* is in good agreement with the figures obtained with isolated nerve fibers.

When DFP is injected into rabbits, the enzyme is always present in the brain of surviving animals, whereas, it has disappeared in the others (Nachmansohn and Feld, '47). In striking contrast to the inactivation of acetylcholinesterase in the animals which died, respiration, glycolysis, adenosinetriphosphatase, succinic dehydrogenase, choline acetylase, and other enzymes tested were not at all affected. These findings show that the high toxicity of DFP must be attributed to its action upon the acetylcholinesterase system.

It has been objected that the concentrations used to abolish conduction of isolated nerve fibers are high, 1 to 2 mg per ml; whereas, in solution a concentration of about 1 μg per ml inactivates the enzyme. However, only the concentration at the site of action is relevant. Surrounding membranes may form a barrier and either prevent or slow down the penetration. In experiments with the giant axon of squid the concentration of DFP inside the axon was found to be of the order of magnitude of 1 μg at the time when the action potential disappeared.

The block of conduction by inhibitors of acetylcholinesterase has been demonstrated with all types of nerves — vertebrate and invertebrate, motor and sensory, pre- and postsynaptic fibers of sympathetic ganglia, and with muscle. Seaman and Houlihan ('51) have recently demonstrated that inhibitors of acetylcholinesterase stop the ciliary movements of *Tetrahymena geleii* S and that the movements are restored when the inhibitors are removed. The esterase is an acetylcholinesterase and is localized in the pellicle where the cilia are located (Seaman, '51).

The question of the essentiality of acetylcholinesterase in conduction has found an unequivocal answer by the use of inhibitors and especially of DFP. Whereas, the first observations on the effect of DFP appeared to be a challenge to the postulate of an essential role of acetylcholinesterase in conduction, the view emerged from the later studies stronger than before.

The studies on the active surface of the enzyme mentioned before have thrown new light on the mechanism of the action of alkylphosphates. It was long believed generally, that alkylphosphates irreversibly destroy the enzyme but this view has to be corrected on the basis of recent studies. The alkylphosphates, that are active inhibitors, are attacked by the enzyme in a nucleophilic substitution reaction (Wilson and Bergmann, '50a). The part of inhibitor entering the covalent bond with the enzyme is a phosphonium ion of the structure $[(RO)_2\ P=O]^+$. Whereas, in other phosphates a C-O bond is broken during hydrolysis, in this case a P-O bond is broken. A phosphorylated enzyme is formed as intermediate instead of the acylated enzyme. A reversal appeared possible by reaction with nucleophilic reagents. Complete reversal of the inhibition has indeed been obtained, slowly with water, more rapidly by reaction with choline, and still faster with hydroxylamine (Wilson, '51).

ENERGY SOURCES OF BIOELECTRIC POTENTIALS

A material of great value for correlating the electric and chemical events of conduction for establishing the sequence of the chemical reactions and energy transformations and for integrating acetylcholine into the metabolic cycle of the nerve cell proved to be the electric organ of fish. The really important feature of the organ is the generally accepted fact that the nature of the discharge is identical with the action potential of nerve and muscle. The electric organ is formed by plates arranged in columns. The plates are the cellular units. The potential difference developed by a single plate is about 0.1 volt, which is of the same order of magnitude as that found in ordinary nerves. It is only the arrangement of these plates in series, like in the Voltaic pile, by which these organs are distinguished. The electric organ has phylogenetically evolved from muscle. The electric plates are considered to be homologous to the motor end plate, at least functionally. Observations of Göpfert and Schaefer ('37) and those of Eccles and his associates (Eccles, '46) have revealed the existence of a special end-plate potential generated most likely in the postsynaptic membrane. The discharge in electric tissue may be homologous to the end-plate potential.

An extraordinarily high concentration of acetylcholinesterase is present in electric tissue. One kilogram of electric organ may hydrolyze 2 to 3 kg of acetylcholine per hour. The high concentration appears particularly pertinent in view of the high water (92%) and low protein content (2%) of these organs. A striking parallelism exists between the concentration of acetylcholinesterase and the voltage and number of plates per centimeter. The relationship between physical event and enzyme concentration has been tested on a great number of specimens of *Electrophorus electricus,* covering the range from 0.5–22 volts per centimeter (Nachmansohn, Coates, and Rothenberg, '46). In figure 5 voltage per centimeter is plotted against acetylcholinesterase concentration. The resulting line calculated by the method of least squares goes virtually through zero, indicating a ''direct proportionality'' between voltage per centimeter and acetylcholinesterase concentration. The finding is particularly significant in view of the striking contrast with the even distribution of other enzymes like respiratory, and glycolytic, adenosinetriphosphatase and other compounds tested.

The direct proportionality between physical and chemical processes in electric tissue suggests their interdependence and close association. Still more information has been obtained, with the use of electric tissue, about the sequence of chemical reactions associated with the electrical manifestations. The most readily available source of energy for endergonic processes in the living cell is the energy of phosphorylated compounds. It is today generally assumed that, in the elementary process of contraction, adenosinetriphosphate (ATP) reacts with the muscle proteins. Phosphocreatine acts as a storehouse for high-energy phosphorylating adenosinediphosphate (ADP) and creatine.

The chemical energy released by the breakdown of phosphocreatine was found to be adequate to account for the total electric energy released by the action potential in *Electrophorus electricus* (Nachmansohn et al., '43, '46). It is safe to assume that the same sequence occurs in nerve as in muscle and that the breakdown of ATP precedes that of phosphocreatine. However, it appeared unlikely for many reasons that ATP breakdown is the primary reaction connected with conduction and acts on the protein of the active membrane.

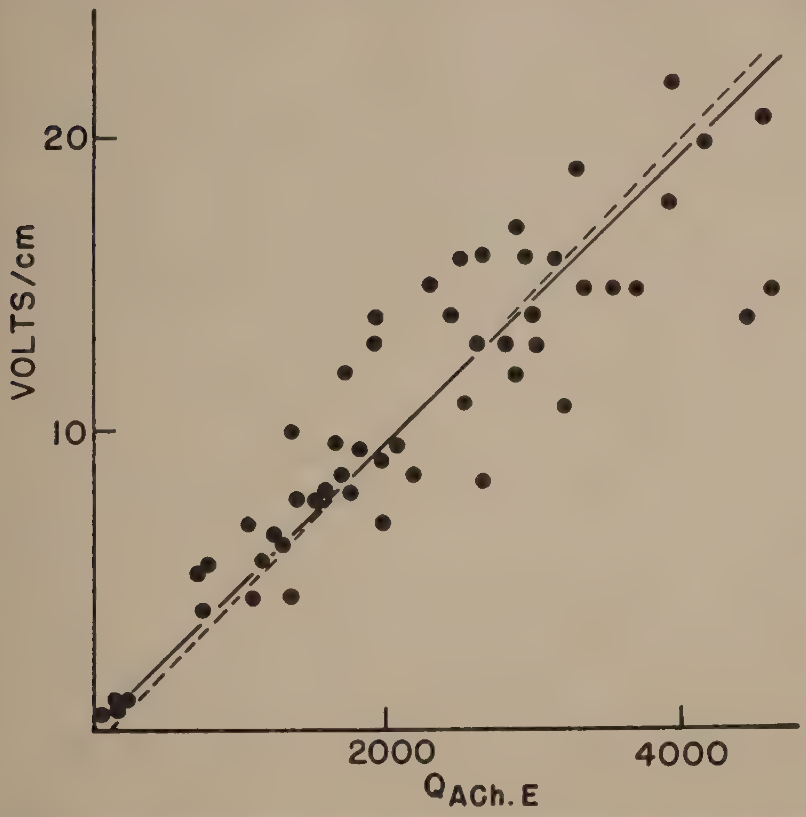

Fig. 5 Direct proportionality between voltage per centimeter and acetylcholinesterase concentration in the electric tissue of *Electrophorus electricus*. Dotted line calculated from data with the method of least squares; solid line calculated on assumption that line goes through zero point (Nachmansohn et al., '46).

Among other obstacles the greatest difficulty is the time factor. The turnover number of adenosinetriphosphatase is 8000 compared to 20,000,000 for acetylcholinesterase. As mentioned before, ionic concentration gradients are the primary source of energy for the electromotive force of the action potential. If, as many indications seemed to suggest, the release of acetylcholine is the trigger which makes the potential source of energy effective and the hydrolysis of acetylcholine is required for the restoration of the resting condition of the protein of the active membrane, this process should occur prior to the breakdown of ATP. The latter would then be the recovery process supplying energy for the resynthesis of acetylcholine hydrolyzed during activity. In agreement with this assumption an enzyme system, called choline acetylase, was extracted from electric tissue and brain which in cell free solution was capable of acetylating choline using the energy of ATP (Nachmansohn and Machado, '43). These investigations made possible the integration of acetylcholine into the metabolic cycle of the nerve cell. Figure 6 illustrates how the sequence of reactions connected with the passage of the nerve impulse may be pictured on the basis of available evidence. The ester must be assumed to be present in an inactive form, bound to protein or lipoprotein, and protected against the action of acetylcholinesterase. Even for the less active ATP it is today generally assumed that it is present in muscle in bound form. When a stimulus reaches the membrane the ester will be released from its bound form. The release may be effected by ions, possibly hydrogen ions, activated by the flow of current. The free ester acts upon the receptor protein. Having produced its effect, the free ester is hydrolyzed by acetylcholinesterase into its two inactive components. The action of the free ester is hereby stopped and the restoration of the membrane to its original condition is initiated. During the recovery period the choline acetylating system resynthesizes acetylcholine. The other steps are, as indicated in figure 6, the same as known from the metabolic cycle in muscular contraction. Except for the first steps involving the interaction with the protein the two cycles are most likely fundamentally the same. In view of the basic difference between conduction and contraction it is not surprising that the first steps differ basically. The wheel driving the process of contraction must be more powerful but may be slower than that

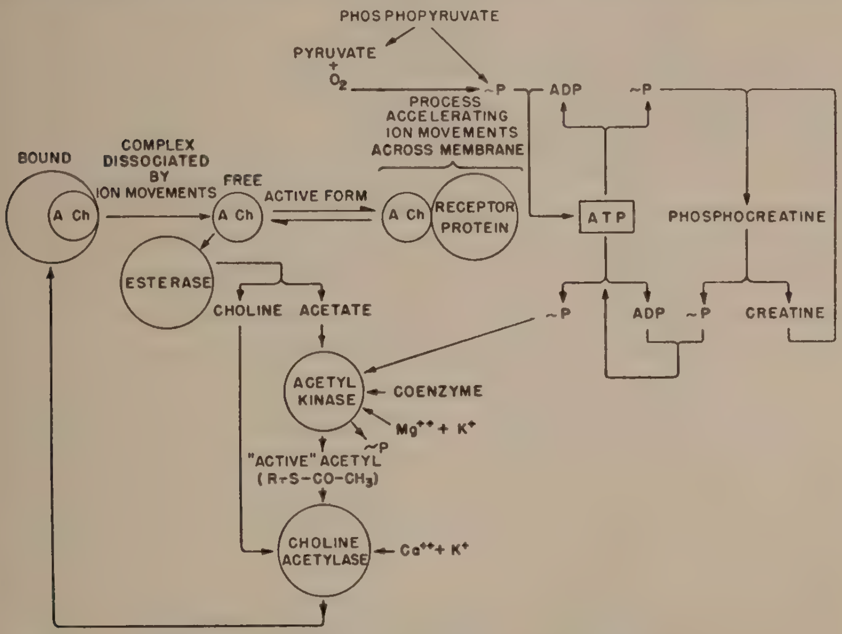

Fig. 6 Integration of the release, hydrolysis, and synthesis of acetylcholine into the metabolic cycle of the nerve cell. ~ P denotes high energy phosphate (Nachmansohn et al., '51).

driving the process of conduction. In the series of successive metabolic energy gears, that of acetylcholine is the fastest and releases suddenly the energy accumulated by the slowly built up ionic concentration gradients.

THE ACETYLATION OF CHOLINE

The discovery that acetylation of choline uses the energy of ATP initiated extensive investigations on the mechanism of acetylation generally. The importance of acetic acid in animal metabolism has been established during the last decade by the use of isotope techniques. Many of the most important cell constituents were found to use the acetate molecule as building stone. These investigations have established the paramount importance of acetate in cellular metabolism.

The finding that ATP provides the energy for the acetylation of choline was soon extended to various types of acetylation. Lipmann and his associates showed that acetylation of sulfanilamide occurs under similar conditions. This showed that the same pathway may be used for the formation of O-acyl and N-acyl groups (Lipmann, '45). Stern and Ochoa ('49) demonstrated that the condensation of oxaloacetate and acetate to citric acid through the methyl groups may be achieved with the energy of ATP.

The acetylating system is rather complex. During the last few years considerable progress has been achieved in the analysis of the process of acetylation by the work of various laboratories. The system requires a coenzyme (called A for acetylation) demonstrated simultaneously and independently by Lipton ('46), Lipmann and Kaplan ('46), and Nachmansohn and Berman ('46). At least two enzymatic steps are involved. In the first, coenzyme A is acetylated by an enzyme which in animal tissue requires ATP; whereas, in bacteria, there is an enzyme which may use acetylphosphate instead of ATP for the acetylation of coenzyme A. The acetylated coenzyme A acts as an acetate donor in the presence of the substrate to which the acetyl group is transferred. The enzyme catalyzing this step is specific for the acceptor. The choline acetylase, e.g., is the enzyme specific for the transfer of acetate from acetyl-coenzyme A to choline.

Purification of coenzyme A has revealed that it contains pantothenic acid (Lipmann and Feld, '47). Lynen and Reichert ('51) have recently obtained from yeast an active acetate which is the acetylated coenzyme. The coenzyme is acetylated in the sulfhydryl group, forming $R\text{-}S\text{-}CO\text{-}CH_3$, probably an acetyl-β-mercaptoethylamine attached to the β-alanine of the pantothenic acid, as the work of Snell et al. ('50) seems to indicate. Lynen and Reichert's startling discovery has revealed a new type of energy-rich compound which may be of great importance in biological systems. Recently it has been shown that thiolacetate may replace ATP acetate (Nachmansohn et al., '52) in the enzymic acetylation of coenzyme A. In these observations the importance of thiol groups for acetylation has been demonstrated in various ways. This recent development is of obvious importance for our discussion and I will return to it later.

SIMILARITIES AND DIFFERENCES OF AXONAL CONDUCTION AND SYNAPTIC TRANSMISSION

The evidence of an essential role of the esterase-acetylcholine system in the generation of action potentials conducting the nerve impulse naturally reopened the question of the role of acetylcholine in synaptic transmission. For a satisfactory answer it seemed necessary to reconcile the earlier findings, on which the hypothesis of neurohumoral transmission was built, with the contradictory conclusions based on the electrical signs and with the biochemical data obtained during the last 14 years.

During the last few years a great amount of evidence has accumulated which readily explains the earlier findings with acetylcholine by the existence of a structural barrier surrounding the axon. This barrier is impervious to acetylcholine and other methylated quaternary ammonium salts. Therefore, the action of acetylcholine applied from the outside and the leakage of ester released during activity are limited to synaptic junctions, which are either less or not at all protected against this type of compound. When giant axons of squid were exposed to sea water containing high concentrations of acetylcholine labeled with isotopic nitrogen, only traces of N^{15} were found in the extruded axoplasm after prolonged exposure. In contrast, a tertiary amine labeled in the same way penetrates readily, and equilibrium is reached within 60 minutes between the concentration inside the axon and its outer environment (Rothenberg et al., '48).

It could be argued that the ester, even if it does not penetrate, may act on the active surface from outside assuming that the structural barrier is identical with the active surface. Only, if a membrane outside the active surface acts as a barrier, acetylcholine would not be able to reach the active surface. In striking contrast to the effect of eserine and various other inhibitors of acetylcholinesterase, prostigmine does not affect conduction. Prostigmine is a methylated quaternary ammonium salt and, in solution is as potent as eserine as an inhibitor of the enzyme. In contrast to eserine and the other inhibitors which were effective and were found in the extruded axoplasm, prostigmine did not penetrate into the interior (Bullock et al., '46). The experiments show that the barrier protecting the active surface against methylated quaternary ammonium salts must be outside the active surface.

These results have thrown new light on the famous observation of Claude Bernard that the pharmacodynamic action of curare is limited to the neuromuscular junction, whereas, nerve and muscle fiber are not affected. Curare is a methylated diquaternary ammonium salt. The active principle appears to be the presence of two cationic nitrogens at a distance of 13–15 A (Barlow and Ing, '48; Kimura, Unna, and Pfeiffer, '49). Bovet and Bovet-Nitti ('48, '49) were the first to realize the structural analogy with two choline derivatives linked together, suggesting that the action of those compounds is based upon competition with the esterase-acetylcholine system. There is good evidence that the effect of curare and similar compounds must be attributed, not to an inhibition of acetylcholinesterase, but mainly to an action upon the receptor protein.

If a structural barrier prevents acetylcholine from penetrating into the interior and reaching the active surface it must certainly be expected that the diquaternary ammonium salts are still less able to penetrate the barrier. However, if the view is correct that acetylcholine has the same function in conduction along the axon as it has in the transmission across the synapse, compounds with two cationic nitrogens at a distance of 14 A should block conduction, provided their structure is modified in a way to make penetration possible. Stilbamidine, a diguanidine derivative, has two cationic nitrogens at a distance of about 13 to 14 A. In contrast to the methylated quaternary ammonium salts it is not completely dissociated; at neutral pH a small fraction, about 1%, is free base. Stilbamidine inhibits acetylcholinesterase at a concentration similar to that of curare. It blocks neuromuscular transmission in about the same concentration as curare. Applied to the axon it also blocks conduction. The concentration required is higher since only a small portion is in a base form (Bergmann et al., '50b). This observation shows, that the pharmacodynamic action of curare and similar substances is, like that of acetylcholine, limited to the neuromuscular junction only because a structural barrier impervious to these compounds surrounds the fibers.

Since acetylcholine can reach the active surface only at the synaptic junction, it must be expected that only there can it leak into the perfusion fluid. This does not imply that during activity acetylcholine is released only at the junction. In 1933, Calabro showed that, if the vagus is cut and stimulated, acetylcholine is released at the cut end (Calabro, '33). Obviously the barrier has been removed by the cutting. Calabro's important observations were confirmed and extended by various investigators, especially Bergami ('36a, b; Bergami et al., '36), who demonstrated the same phenomenon with motor nerves, and Brecht and Corsten ('41), who used sensory nerves. These observations, considered for a long time as artifacts, actually show that during activity acetylcholine is released along the axon as well as at the synapse and that there is no justification for considering the release of acetylcholine as an event peculiar to the synapse.

More than ten years have elapsed since the Symposium on the Synapse mentioned before. Much new information has been obtained about the electric characteristics of transmission across natural and artificial synapses. The steadily increasing evidence supports Erlanger's conclusion that the mechanism of conduction along axons and transmission across synapses is basically the same. The propagating agent for both cases is the flow of current. In an excellent and interesting review, Bullock ('51) has critically discussed and evaluated the most recent progress in this respect.

If electrophysiologists do not find any justification for assuming two basically different mechanisms, the chemical data certainly do not justify such a theory. There remains not a single fact supporting the idea of chemical mediation: that acetylcholine assumes a function at the synapse entirely different from that in the axon and, being released from the nerve ending, replaces the flow of current as a chemical mediator. However, although the propagating agent is the electric current in conduction as well as in transmission, the essentiality of acetylcholine in both processes cannot be questioned. If the ester is essential for the generation of the flow of current, its release within the membrane of the nerve ending will be essential for the generation of the current which propagates the impulse from the ending to the postsynaptic membrane. If we accept the postulate of Keith Lucas and Adrian (Lucas, '17) that the small electric currents propagating the impulse along the axon require locally supplied energy, this postulate must apply to the synaptic junction when the current reaches the second unit. If a trigger mechanism must be postulated at successive points activated by the current to make the potential source of energy effective, such a trigger mechanism must be present in the postsynaptic membrane. Interference with the system will produce interference with the electrical events at all sites.

A fundamental role of scientific thinking requires that one should not assume two different principles without necessity. The structural differentiation forms the basic difference between axon and synaptic junction, not the physiochemical mechanism of propagation. This view reconciles easily all the earlier observations on which the hypothesis of chemical mediation was based, with the conclusions based on biophysical and biochemical data.

If the primary event in conduction is the change of permeability of the active membrane to sodium and acetylcholine is responsible for the process, the ester should have an electrogenic effect. This postulate cannot be tested with axons, in view of the protective barrier. The electric tissue, however, is suitable for such a demonstration since the discharge here is homologous to the end-plate potential and acetylcholine should be able to reach the active surface. If acetylcholine is injected intra-arterially into electric tissue an electrogenic action is observed. The potential changes produced are similar to the natural discharge. However, relatively large amounts are necessary for obtaining these effects even in presence of eserine. Figure 7 shows effects produced by increasing amounts of acetylcholine injected into the electric organ of *Torpedo marmorata* (Feldberg et al., '40). The experiments

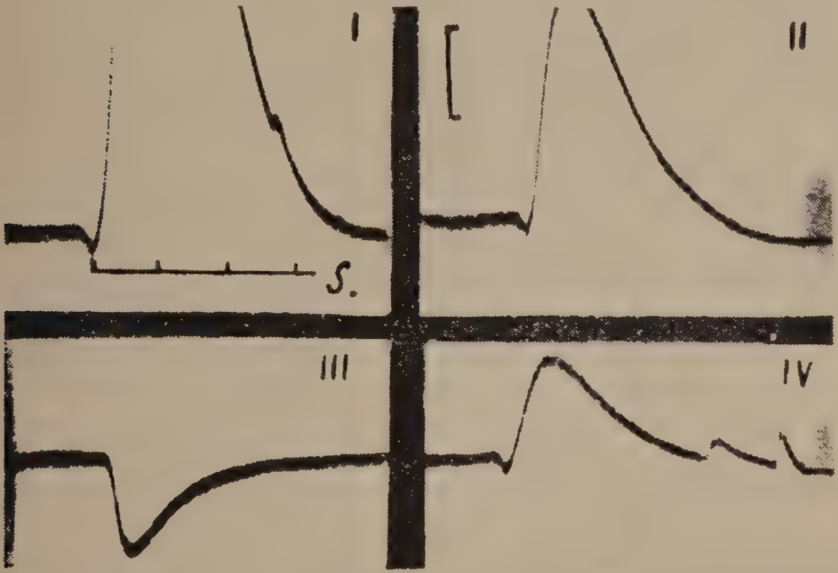

Fig. 7 Electrogenic action of acetylcholine. The potential changes are produced by intra-arterial injection of acetylcholine into the electric organ of *Torpedo marmorata* in presence of eserine. I, II, and IV correspond to the injection of 10, 5, and 2.5 μg of acetylcholine; at III only perfusion fluid was injected. 0.5 mv indicated at II. Time in seconds.

indicate that the ester may produce alterations of the membrane which generate flow of current. This fact supports the assumption that the release of acetylcholine precedes or coincides with the flow of current and makes it difficult to assume that this event occurs in the recovery period. In that case it would be hard to understand how the compound produces current. The electrogenic action of acetylcholine is particularly pertinent in connection with the potentially high speed of the reaction and other aspects of the system described before. Other compounds may be able to produce a discharge but only in the case of acetylcholine have the many prerequisites been established for assuming the ester to be the natural reactant responsible for the permeability change.

Although the potential changes resemble the normal discharge there is a more striking contrast in two respects: (1) the voltage is small — about 3 mv was observed after injection of 10 μg of acetylcholine; (2) the duration is 3 seconds, which is a thousandfold increase compared with the normal action potential. A quantitative evaluation is impossible, since the number of units in series reached by the injection is uncertain and the method is crude compared with the effects which might be expected if the compound were released from the nerve ending. The discrepancy as to duration and strength appears, however, enormous and beyond all proportion. If, however, it is assumed that the release and removal of the ester are intracellular events which occur within the active membrane and do not involve any diffusion, the action may be infinitely faster and more powerful. If the ester is present in the active membrane in a bound form the flow of current activating the movement of ions may lead to a release of the bound to a free form by breaking the bond between the ester and protein. If, for instance, the carbonyl carbon is bound to a nucleophilic group in the protein, hydrogen ions may break this bond and in this way release the ester. The free form of acetylcholine may act upon the receptor protein at a distance of a few Angstroms. The free form will be in equilibrium with the receptor protein. The ester will be open to attack by the esterase and the hydrolysis will initiate the restoration of the resting condition.

Addendum

(February 20, 1952)

As discussed above, the action of acetylcholine, curare, and other quaternary ammonium salts, when applied externally, is limited to synaptic junctions because of a permeability barrier surrounding the fibers. This barrier prevents these quaternary ammonium salts from entering the interior of the axon and acting upon the active surface. It appeared desirable to demonstrate that these compounds, when brought into the interior of the axon, affect axonal conduction. Such experiments are technically rather difficult and early attempts in 1945–46 were unsuccessful. However, a few months after this lecture was given, such a demonstration was achieved (Grundfest et al., '52). With the aid of a micromanipulator, a micropipette was inserted and a few cubic millimeters of fluid were injected into the giant axon of squid without damage to excitation of or propagation in the fiber.

When 0.1 μg of prostigmine was injected in this way, conduction was blocked within 10 to 30 seconds, although the compound is completely inactive when applied externally in 0.01 M concentration 3 mg per ml). About 1 μg of eserine, i.e., ten times as much as in the case of prostigmine, is required to produce a block in the same length of time, although eserine blocks conduction when applied from the outside in 0.001 M concentration. An amount of 0.00001 μg of D-tubocurarine was found to produce block of axonal conduction. The effect on axonal conduction is thus many thousand times as powerful as that on neuromuscular transmission. The same is true for acetylcholine which affects conduction in concentrations much lower than those which affect synaptic transmission.

The experiments are a new and striking demonstration for the view, established by so many physical and chemical facts, that the mechanism responsible for the propagation of the nerve impulse along axon and across synapses is basically the same. (End of Addendum.)

EFFECTS OF X IRRADIATION ON THE SQUID AXON

Having discussed the present concepts of the elementary chemical and physical events underlying the function of nerve, I would like to turn now to the problem of how these processes may be affected by radiation energy. Very little information is at present available and I would like therefore to emphasize mainly a few lines of approach which seem to me promising.

As we have seen, ionic concentration gradients between the inside of the axon and its outer environment are the primary source of energy supplying the electromotive force for the electric currents propagating the impulse. It appeared possible that a disturbance reaching the active membrane would result in an alteration affecting the rate of ion flow in both directions in a way similar to that of an impulse traveling along the axon.

Having determined, with the aid of Na^{24}, the rate of sodium entrance into the interior of the giant axon of squid, the effects of high intensity X irradiation and the movements of this ion species have been studied. The nerves were irradiated with 50,000 and 125,000 r and immediately after irradiation exposed to artificial sea water (Rothenberg, '50). Part of $Na^{23}Cl$ was replaced by $Na^{24}Cl$. With 125,000 r the rate of penetration of sodium increased quite markedly. With 50,000 r the increase was smaller but still significant. Conduction was unimpaired, so there was no apparent damage of function.

It should be noted that these studies were carried out immediately after irradiation. The marked alterations in membrane permeability observed are interesting in spite of the high doses applied. It is possible that a more pronounced effect would be evident with smaller doses of irradiation if longer periods of time were permitted to elapse between irradiation and exposure to radioactive ions. Strong effects of exposure to radiation are known to occur frequently after prolonged periods of time. It is in any case significant that increased permeability to ions has been demonstrated as the result of X irradiation.

It seems to me that the observations have gained in interest by the results obtained with red blood cells, which have been presented by Sheppard ('50), at this meeting, and at the Society of General Physiologists. The ionic exchange across the cell boundary in red blood cells is apparently affected in a way similar to that in the nerve following the exposure to X radiation. The doses required are of the same order of magnitude. Physiologists have long observed that certain similarities, in respect to permeability, exist between properties of the red blood cell and those of nerve fibers. In view of the role of the esterase-acetylcholine system in the alterations of the active neuronal membrane it is noteworthy that the same system is found in red blood cells. They are the only cells except conductive tissue which contain acetylcholinesterase. The enzyme is localized exclusively in the surface. The red blood cells contain appreciable amounts of choline acetylase (unpublished observations). Greig and her associates have been accumulating evidence during the last few years that acetylcholine and acetylcholine-like compounds and inhibitors of acetylcholinesterase affect the permeability of red blood cells (Greig and Holland, '49; Holland and Greig, '50). This seems to be an interesting working hypothesis and I have been following this work with great interest. Sheppard's data point in the same direction. However, I would like to caution against some of the generalizations. For instance, I cannot accept Greig's statement that the esterase-acetylcholine system is associated with selective permeability of living cells (Greig et al., '50). Besides many other objections, the prerequisite for such an assumption would be the presence of acetylcholinesterase in all cells, whereas, its absence excludes this possibility.

PERMITTIVITY OF BRAIN CORTEX SLICES

The changes of permeability of the active neuronal membrane to sodium and potassium is only one aspect of the permittivity of the neuron to various substances. For the elementary process of conduction this special property is of paramount importance. However, like all cellular boundaries that of the neuron must be endowed with the ability to permit or to prevent the entrance and leakage of various compounds and metabolites. This makes it possible to eliminate waste products and to permit the entrance of substances important for the energy requirements and other vital functions of the cell. There is increasing evidence that the transportation of quite a few compounds across membranes requires energy and is associated with chemical reactions. The introduction of isotopes as a research tool in biology has opened new pathways to approach the problem. It made it possible to follow the rate of entrance of various compounds and to analyze the effect of chemical and physical factors upon this rate, although the underlying mechanism, i.e., the chemical reactions responsible for the transport, are of a complex nature and require a different approach.

In view of the findings that exposure to X radiation affects the permeability of the active membrane to ion movements, it appears of interest to test whether this action is specific or whether the permeability to other compounds is altered as the result of exposure. Experiments on the entrance of amino acids labeled with isotopic nitrogen (N^{15}) into the giant axon of squid have been initiated and the effects of X irradiation on this process are being studied. The work is still in progress and some of the data are not yet ready for publication. Due to its simplicity this material is extremely favorable and unique for certain aspects of the problem. However, a great difficulty is the limited amount available and the very short period of the year during which the material can be obtained.

Even not taking into account the technical difficulties the giant axon of squid is only a special case. Additional information is necessary about the more complex situation prevailing in other nerve tissue. Furthermore, we have seen that axons are surrounded by membranes which form a structural barrier for certain compounds, in which these structures are absent; cell bodies are more susceptible to chemical and also physical agents. Finally, certain vital functions which most likely are absent in the axon must be attributed to the cell body.

Investigations have been initiated on the rate of penetration of amino acids labeled with N^{15} into the brain cells and on the factors affecting this process (Korey and Mitchell, '51). The use of amino acids to determine the nature of permittivity and of surface and metabolic relationships has various possibilities. A great variety of structurally distinct amino acids are available which differ in molecular volume, carbon chain links and polar groups, and are useful therefore for exploring membrane characteristics.

Slices of rabbit brain cortex were used as material. The time of exposure was usually limited to maintain optimal conditions as indicated by the rate of respiration. The use of the Stadie-Riggs instrument ('44) secured uniform thickness of the slices in all experiments.

One important technical difficulty in permeability studies with biological material is the problem of extracellular space. With the use of inulin (Harrison, '42) the total volume of interstitial space was determined and, simultaneously, the time required to equilibrate the intercellular space with the outside fluid. Figure 8 gives the results obtained. Only the extreme values are presented. The volume of the intercellular space varied between 19 and 34% of the total volume, with an average of about 30%. The time required for attaining the equilibrium was about 5–10 minutes depending upon the usual factors determining diffusion. Any substance of the outside fluid entering the cells will first fill this interstitial space, and this process must be taken into account in the evaluation of all observations.

The rate of penetration of glycine and aspartic acid has been studied. The rate of the initial period is probably determined mainly by the entrance of the amino acids into the interstitial space. The diffusion of these two amino acids into the interstitial space takes place within 3–5 minutes at 37°C. However, it takes another 5–10 minutes before a persistent but less rapid increase of the concentration of the amino acids into the slice becomes apparent. This increase represents the intracellular uptake. From the data it is possible to calculate the ratio of the intracellular to that of the extracellular fraction of amino acids which entered. The figures indicate that, altogether, most of the glycine which was present in the slice after the first 5 minutes must be attributed to the fraction which entered by diffusion into the free space. A small fraction of the total (10–20%) entered the cells during this period. The rates for the diffusion and penetration of glycine and aspartic acid appear in principle

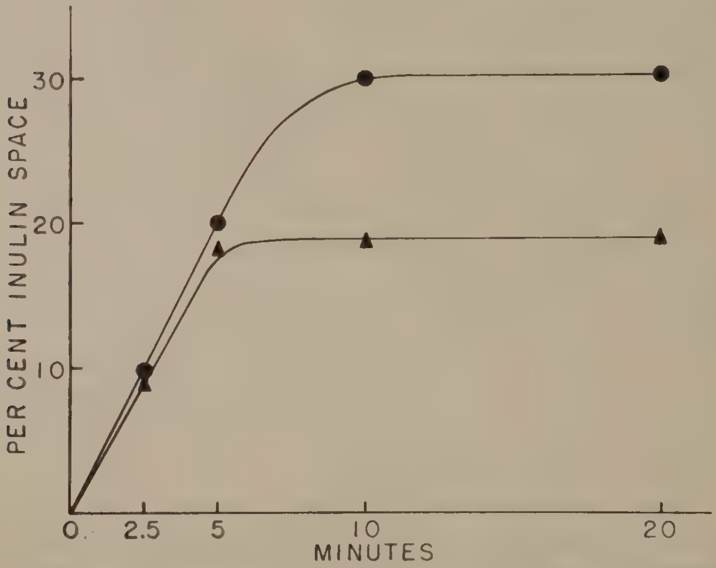

Fig. 8 The rate of penetration of inulin into cortical slices of rabbit brain indicating the percentage of the intercellular space. The two curves represent the extreme values between which the percentage varied (Korey and Mitchell, '51).

similar (fig. 9) although, in the case of aspartic acid, equilibrium between the external fluid and the tissue slice was obtained in only a few instances and required a period of time more than twice as long as in the case of glycine.

In spite of the apparent similarity of the penetration of the two amino acids into the cortical cells there are, however, different mechanisms involved. The rate of entrance of glycine during the period of exposure from 10–40 minutes, i.e., after the diffusion period into the intercellular space, has a Q_{10} of the order of 1. This indicates that the intracellular penetration into the cells is not associated with reactions which have a high rate of activation. In contrast, the Q_{10} for the rate of penetration of aspartic acid is 2.4 (fig. 10). This entrance is apparently linked to an active process. Moreover, when respiration is inhibited by cyanide the pene-

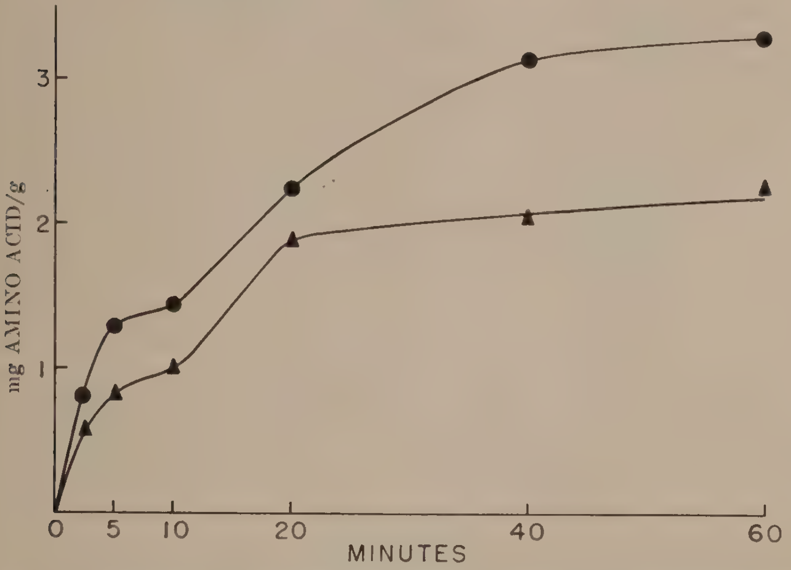

Fig. 9 Comparison of the penetration rates of glycine (▲) and aspartic (●) acid into cortical slices. Average of 8 experiments. The shapes of the curves are similar. In the slices exposed to glycine, equilibrium was reached in about 40 minutes; whereas with aspartic acid, it was not attained within 60 minutes (Korey and Mitchell, '51).

tration of glycine is not affected, whereas, that of aspartic acid decreases by 25% (fig. 11). This markedly reduced rate in absence of respiration is further evidence for the dependence of penetration of aspartic acid upon an active process. In contrast to the effect on ionic movements, inhibitors of acetylcholinesterase do not affect the rate of entrance of the

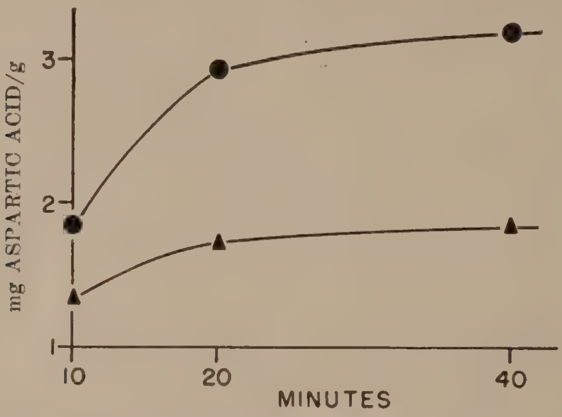

Fig. 10 The rate of intracellular penetration of aspartic acid as a function of temperature. Q_{10}, measured between 10 and 40 minutes, is about 2.4. ● = 37°C. ▲ = 23°C. (Korey and Mitchell, '51).

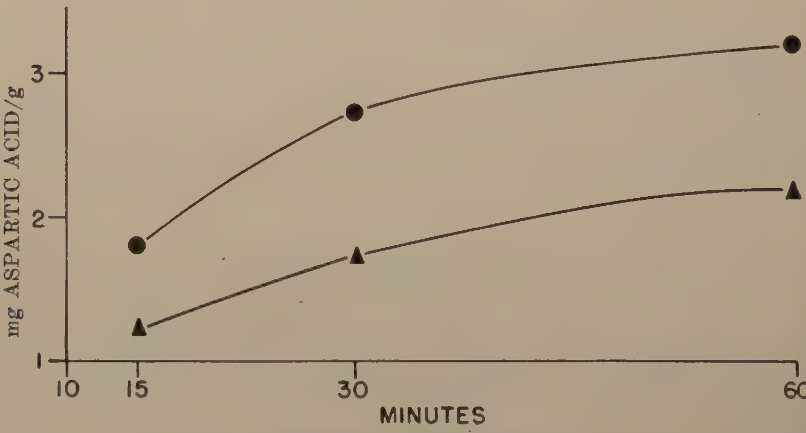

Fig. 11 The effect of inhibition of respiration by hydrogen cyanide (▲) (0.02 *M* final concentration) upon the rate of penetration of aspartic acid. CO (●) = controls. t = 37°C. (Korey and Mitchell, '51).

two amino acids. The effect of X irradiation is being investigated; the data are not yet ready for presentation.

It is of interest to compare the rate of entrance of glycine and aspartic acid into cortical cells with that into the giant axon of squid. In 30 minutes at 23°C., 16% of the external concentration of glycine and 5% of aspartic acid are found within the axoplasm (Korey, '50). At corresponding temperatures and concentrations relative to the total molarity of the external fluid, 40% of glycine and 13% of aspartic acid would be intracellular. The ratio of glycine to aspartic acid is in both cases about 3:1. However, the absolute amounts found are markedly greater in the cell than in the axon. The results are part of the general problem of whether the penetration into the cell body occurs at a higher rate than into the axon. There are many indications that this may be frequently the case; in view of the structural membranes surrounding the axon, this difference is not surprising.

Knowledge of the physical and chemical processes associated with cellular function must form the basis of the understanding of the effects of exposure to radiation energy on cellular mechanisms. As long as medicine was essentially limited to the study of histological changes, investigations of interference with function were difficult. Only relatively severe or more lasting cellular damage and injuries could be detected. The great advance of the last decades has opened many possibilities for studying earliest interference with cellular function. In discussing here permeability of nerve cells in relation to radiation I have restricted myself to only one aspect. There are others of equally great interest. We have seen that the recent discoveries of Lynen and Reichert and the subsequent results of other laboratories have revealed the importance of sulfhydryl groups in coenzyme A and in acetylation generally. Besides, for the acetylation of choline, coenzyme A is essential in the citric acid cycle. Acetate is a building stone for many other vital cell constituents. Since we know that ionizing radiations inactivate sulfhydryl groups, damage of systems involving coenzyme A may be expected.

This will be of special interest in growing cells. Here is a field still completely unexplored. It is an illustration of how each progress in the understanding of fundamental reactions will affect the approach of study of irradiation effects upon biological systems.

ACKNOWLEDGMENTS

It has been a source of great encouragement to our group and others since Dr. Shields Warren recently emphasized the importance of studying the fundamental principles. I take this opportunity to express our thanks to the Atomic Energy Commission for their generous support of these studies. Being in Oak Ridge, I also would like to thank Dr. Paul Aebersold for his frequent assistance in our efforts to obtain the radioactive material. The difficulty in getting the biological material was mentioned. In view of the short half-life time of radioactive sodium and potassium, it was sometimes not easy to bring the giant axons of squid together with the products of the pile from Oak Ridge. The affinity of the two materials seemed to be very small. Dr. Aebersold, by cutting through red tape, frequently improved the Michaelis constant and saved many of our experiments by helping us to get the material in the right time. His efficient and untiring cooperation is greatly appreciated by all of us.

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SOME PROBLEMS IN CONNECTION WITH ION PERMEABILITY OF ERYTHROCYTES

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The problem of the permeability of cells for ions has long fascinated many investigators. Under this stimulus the term "ion permeability" has taken on many meanings. These range from those obtained from studies on the *permeation* of ions through tissues and organ structures, such as skin, gills, kidney tubules, intestine, pia mater, etc., to the meanings obtained by an examination of the *accumulation* of particular ions within a particular cell. In the case of the passage of ions through tissues and organs the functional activity of a complex multicellular structure with respect to the ions is usually under scrutiny. On the other hand the ion accumulation phenomenon may frequently involve events which have occurred during the growth and development of a cell.

Throughout the studies on these phenomena of permeation of ions through tissues and organ structures and of accumulation of ions in cells it has been assumed that the permeability for ions of the plasma membrane of the cell or cells under examination must be an important factor. Very frequently the part played by this factor of permeability relative to the whole phenomenon has not been assessed. Recent excellent reviews on permeability (Teorell, '49; Steinbach, '51) have placed emphasis on the need for a clearer understanding of the term "permeability," but unfortunately these reviewers turn about and use the term to apply to all the situations mentioned above.

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On the other hand, Sheppard ('51) has avoided this ambiguity in a fine résumé of the last 10 years of "potassium and cell physiology."

The foregoing has been mentioned simply to lay stress on the fact that this paper will not deal with these varied phenomena of *permeation* or *accumulation* but will confine itself to an examination of the *permeability* properties of the plasma membrane of mammalian red cells for cations; in particular, potassium and sodium.

As is well known, the normal environment of the red cells of various species of mammals contains sodium in much higher concentrations than potassium (usually in ratio ca. 27 sodium to 1 potassium). On the other hand, the red cells of various species of mammals show wide variation in this ratio of sodium to potassium (e.g., 1 sodium to 9 potassium in the pig and 17 sodium to 1 potassium in the cat).

Most investigators who have studied the problem of the permeability of red cells to potassium and sodium have used the cells of those species of mammals which have high potassium. Relatively few experiments have been performed on the penetration of potassium from an environment enriched with this ion, into red cells of high sodium, low potassium contents (Davson, '40). This paper will deal with red cells of those species of mammals that have a high potassium content.

Any investigation of the rate of penetration of potassium and/or of sodium must be done under conditions where the amount of these ions bound to the nondiffusible hemoglobin within the cell is known or is reduced to a minimum, e.g., at the isoelectric point of the hemoglobin. Under these conditions it has been found that the rate of penetration of potassium from the cell to the environment is precisely balanced by the rate of penetration of sodium from the environment into the cell (Ponder, '51; Sheppard, '51; Parpart and Green, '51). This exact exchange of potassium within the cell for sodium outside the cell has been observed under a wide variety of experimental conditions. The only divergences from this relation are to be found in the observations of Harris ('41), of Ponder ('51), and of Flynn and Maizels ('49). It is noteworthy that the last authors have minimized their earlier work on potassium uptake (Maizels, '49) against a concentration gradient and have laid stress on an active sodium loss from the cells, thereby assuming a normally high impermeability for potassium. All these experiments in which potassium uptake against a concentration gradient was observed have been performed under experimental conditions where the red cells were stored from 4 to 15 days at 4°C. and then, on addition of glucose and subsequent raising of the temperature to 37°C., the uptake was obtained. While no satisfactory explanation of this uptake of potassium has been offered, it is perhaps permissible to speculate as to whether the anomalous volume behavior of red cells associated with "gelation" of hemoglobin (Ponder, '40) or with the "paracrystalline" state (Ponder, '48) might play an active part in these observations on potassium uptake.

In any event, as matters stand at the moment, an exact exchange and exchange rate of potassium within the cell for sodium outside the cell will occur under a wide variety of conditions. These experimental conditions have varied from low (0°C.) to high (37°C.) temperatures; wide pH range (6.0–8.0); low glucose content (0–5 mg %) to high glucose (100–1000 mg %); under the influence of lytic concentrations of simple hemolysins and in the presence of a wide variety of metabolic inhibitors.

The rate of this exchange of red cell potassium for environmental sodium is highly variable. The exchange rate may be exceedingly rapid, so that equilibrium exchange occurs in several minutes (Parpart and Green, '51); or it may be very slow so that equilibrium exchange may take 40 to 50 days (Parpart et al., '47). There is no doubt, therefore, that potassium and sodium ions can pass through the plasma membrane with great ease under some circumstances and only very slowly under other conditions.

How might one interpret information of this sort in relation to the permeability of the plasma membrane for potassium and sodium? The type of interpretation that is needed is one that will explain how the normally slow rate of exchange of potassium and sodium may be speeded up. Several alternative, but not mutually exclusive, hypotheses for this permeability change may now be examined.

Any alteration in the molecular configuration of the plasma membrane could account for these differences in the rate of exchange. Changes in the rate of penetration of compounds into or out of a cell have, of course, been accounted for in this way for a long time. In the specific cation permeability under consideration very little evidence for or against this idea has been available. Indirect evidence in the work of Jacobs and Parpart ('33), Jacobs and Willis ('47), and of Ponder ('51) strongly support this hypothesis. More recently, however, the rapid loss of potassium and equivalent uptake of sodium induced prior to hemolysis in the presence of high lytic concentrations of *n*-butyl alcohol, has been shown to be of a nature that, upon removal of the lytic agent prior to the attainment of equilibrium distribution of potassium and sodium, the cell recovers its normal slow permeability to potassium and sodium (Parpart and Green, '51). These facts argue very strongly for the increased cation permeability caused by a simple lytic agent being due to an alteration in the arrangement of molecules, possibly the lipids bound to proteins (Parpart and Ballentine, '51) in the plasma membrane; and also that such alteration of molecular configuration of the plasma membrane is reversible.

A similar action has been observed with relation to the increasing permeability to nonelectrolytes induced by the action of the enzyme, lipase (Ballentine and Parpart, '40). Green ('51) has demonstrated increased permeability to potassium of red cells under the influence of lipase and of trypsin. In a similar manner Sheppard ('50) and Sheppard and Beyl ('51) have shown that X irradiation of whole blood causes an exchange of red cell potassium for plasma sodium with an equivalent exchange rate. This may be interpreted as a case of a physical agent acting on the plasma membrane and inducing a slight increase in cation permeability. As yet, no studies on the reversibility of the effect of X rays or enzymes on cation permeability have been made. It should be pointed out that the above statements with respect to X-ray effects are based on data given in table 1 of Sheppard and Beyl ('51). They do present data on the effect of X irradiation on uptake of K^{42} and find no difference compared with nonirradiated controls. Two interpretations of this fact suggest themselves; first, though studies on the uptake of K^{42} may measure the permeability rate from plasma into cell, it by no means establishes the rate of penetration of potassium from cell to plasma, which is the situation upon which the above interpretation of their table 1 was based. Secondly, and less probable, is the possibility that the radiations of the K^{42} acting on control red cells have an effect on the control rate of K^{42} uptake, bringing it to that of X-irradiated cells. There is very real need for data on the rate of loss of K^{42} from red cells.

Another factor which affects the permeability of the red cell to potassium and sodium ions is the temperature at which the blood is maintained. Parpart et al. ('47) found 5°–7°C. the temperatures at which the lowest rate of potassium loss from red cells was obtained. Below and above this temperature range, a marked increase in the rate of loss of potassium in exchange for sodium was observed in all of a large series of experiments. The slowing of the rate of loss of potassium from the red cells as the temperature of the system is lowered occurs in spite of a coincident decrease in the binding of potassium by the nondiffusible hemoglobin in the red cell. This influence of decreasing temperature on the rate of potassium loss may be interpreted as a decrease of the permeability of the plasma membrane for potassium ions. Below 5°C., however, a reversal of this action takes place. It should also be pointed out that this decrease in the rate of loss of potassium ions from red cells as the temperature is lowered is accompanied by a marked decrease in the rate of utilization of glucose by these cells; a fact which is at variance with current ideas that increased rate of glucose utilization is accompanied by a decreased rate of loss of potassium from the cell.

Another environmental factor that markedly influences the permeability of the plasma membrane for potassium is the hydrogen ion concentration of the environment (Parpart et al., '47; and Parpart and Green, '50). These papers showed that a pH in the range of the isoelectric point of hemoglobin (6.6–6.8) will very markedly slow the rate of loss of potassium from red cells. Changes in pH, to pH 6 or to pH 7.5, cause a marked increase in the rate of loss of potassium. This was observed at 7° and at 37°C. in the red cells of rabbit and man.

The concentration of glucose also affects the permeability of red cells for potassium (Parpart et al., '47). At constant temperature and at pH 7.0, red cells of man exhibit a slower and almost constant loss of potassium when the glucose concentration ranges between 500 and 2000 mg % than at lower or higher concentrations. Concentrations of glucose above 750 mg % or less than 250 mg % greatly alter the osmotic resistance of the red cells.

Still another factor that alters the permeability of red cells to potassium is the addition of various agents, classed as metabolic inhibitors. Iodoacetate, fluoride, *p*-chloromercuric benzoate, and cyanide are typical inhibitors that have been shown to increase the permeability of red cells for potassium (Green and Parpart, '50). Do these metabolic poisons act through their effect on glycolysis within the red cell and/or action on the plasma membrane? Our evidence on the increased permeability to potassium induced by these poisons in red cells whose glucose utilization has been reduced to zero prior to adding the poisons, strongly supports the idea that these metabolic poisons affect potassium permeability, primarily through their action on the plasma membrane. This is in agreement with the conclusions of Flynn and Maizels ('49) that "iodoacetate damages cells and leads to a fall of potassium."

Lest it be argued that the permeability of the red cell for potassium, as it is usually measured by studies on the average behavior of a population of red cells, may be an expression of wide differences in the initial potassium content of the individual cells of the population, it should be pointed out that, at least for red cells of the rabbit, all the red cells have the same potassium content (Parpart et al., '51).

The permeability of the plasma membrane of mammalian red cells for potassium and for sodium, studied by means of their exchange, shows that this permeability may be so variable as to bring about equilibrium exchange in a few minutes on the one hand or only after many days under another set of conditions. In the case of the red cells which have been put into an environment that induces a rapid cation exchange, the permeability for potassium and for sodium must approach that for water, since these cells undergo a slow volume increase. This volume increase is the resultant of the passage of water into the cell under the influence of the unopposed colloid osmotic pressure of the hemoglobin, since the cell is now freely permeable to cations (Wilbrandt, '41; Jacobs and Willis, '47; Parpart and Green, '50, '51).

The interpretations of the permeability data for potassium and sodium that we have thus far considered have stressed the idea that it is the plasma membrane of the red cell that is the single, most important factor in maintaining the potassium content of the adult cells.

Since accumulation has not yet been observed in mammalian red cells that are kept at 37°C., it still remains to determine how much of a role an accumulation process plays in the maintenance of the potassium content of the cell. Present data (summarized and amplified by Ponder, '50) indicate that under the experimental conditions of storage (2–4 days) of cells at low temperature (4°C.) until some potassium loss has occurred and then return to 37°C. in the presence of glucose, there occurs not only a slower rate of loss of potassium but a slight gain.

It should be pointed out that the rate of loss of potassium at 37°C. and at pH 7.3 of red cells not exposed to low temperature is slower in the presence of added glucose than in the absence of glucose (Parpart and Green, '50). Above and below pH 7.3 this difference on adding glucose decreases and disappears by pH 6.5 and 7.5. This is interpreted as meaning that at a pH around 7.3 the permeability of the plasma membrane for potassium is affected by glucose and/or glucose utilization. This is less or not at all true at pH 6.5 and 7.5; even though the rate of utilization of added glucose by the red cells at these three hydrogen ion concentrations was found to be the same.

It would appear that what is needed is a well-controlled assessment of the relative roles of the permeability of the plasma membrane and of possible potassium accumulation and/or sodium extrusion mechanisms (Flynn and Maizels, '49) participating in maintaining the normal inequality in the distribution of potassium and sodium ions between the red cells and plasma of many species of mammals. We are at present inclined to believe that a normal low permeability to potassium and sodium is the dominant factor.

No attempt has been made to discuss the mechanisms that may be involved in the actual passage or availability for passage of cations from cell to environment, or vice versa. Certainly, the amount of potassium in the cell that may at any one time be in salt linkage with hemoglobin is not a limiting factor, since all the now-available evidence affirms the belief that neither potassium nor sodium preferentially links with hemoglobin. It must be concluded that any potassium or sodium in the cell is available for exchange, and hence for passage across the plasma membrane.

Several mechanisms for the passage of cations through the plasma membrane in a selective manner have been offered and recently summarized (Steinbach, '51). Red cell ghosts have been tested and found to exhibit no preferential pickup of potassium or sodium as demanded from such mechanisms of passage — theories as that of preferential selective exchange (e.g., H^+ in the cell for another cation, Brooks, '37), or preferential solubility in or complex formation with some constituent of the membrane (Osterhout, '32), or a metabolic cycle to provide a source of ion complexing agent.

Certainly the evidence presented in this paper is more consistent with the idea that the molecular barrier of the plasma membrane is the primary factor in determining the ease of passage, the permeability of the cell for cations. As indicated, this molecular barrier may be reversibly altered.

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THE DIRECT EFFECTS OF RADIATION ON ERYTHROCYTES[1]

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FOURTEEN FIGURES

From time to time radiobiologists and experimental radiologists have given passing attention to the effects of ionizing radiation on the mammalian red cell *in vitro*. The radiologist has passed on after satisfying himself that no obvious influence on mature red cells can be attributed to the direct action of clinical doses of X rays. The radiobiolgist has made a mental note to return to the study of red cells after he has finished the interesting cytogenetic investigations of the moment. However, as radiation studies continue, our interest has matured and we wish to become familiar with all the things radiation might do to cells at all dose levels. In this paper the intention is to show why, from this point of view, the red-cell system has certain unique and useful characteristics for radiation study, and to discuss some of our recent findings, as well as to summarize those of earlier workers.

The mature mammalian erythrocyte is possibly the most highly specialized of all animal cells, and has no interesting structural features except perhaps its biconcave shape. To the morphologist this cell is distinguished primarily for the properties it lacks, such as motility and invasiveness, reproductive power, or the ability to ingest foreign particles. Principal among all these is the lack of a nucleus or any nuclear remnants that might be demonstrable by Feulgen staining. Despite this

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high degree of sophistication, the erythrocyte has several properties in common with other living cells. Principal among these is its metabolism. Another property which is of increasing interest to the cell physiologist is its ability to maintain concentration gradients of cations between the cell contents and the surrounding medium. It is the direct effect of X rays on this latter property, uncomplicated by nuclear effects, which will be stressed in this communication. Since the mammalian host suffers profound general disturbance at the radiation doses required, we will be concerned primarily with experiments *in vitro.*

EXPERIMENTS IN VITRO AND THE ENVIRONMENT OF THE RED CELL

Since the erythrocyte is less readily adaptable to environmental disturbances than most animal cells, we must briefly consider environmental factors. For experiments *in vitro* nature has conveniently provided to the investigator a natural culture medium, the plasma, in which cells can be kept at least for a few hours in an apparently physiological condition. The plasma contains the various substances which are required for the health of body cells in general, for example, sugar and phosphates and those anions which participate in the buffer system which maintains the proper pH. Included among the buffers are the plasma proteins which also protect in a different way by exerting a stabilizing influence against small amounts of surface-active substances which might attack the cell envelope. In the body the environment is maintained by the various organs such as the liver which provides the proteins and the energy source, the kidney which removes the waste products and helps to stabilize the pH, and the lung which is a major line of defense against pH disturbances. Outside the body these considerations are important in understanding the factors which are involved in the maintenance of blood for a sufficiently long period to permit studies of cell electrolytes.

SODIUM AND POTASSIUM MOVEMENTS IN RED CELLS

During the last 10 years our conception of the role of sodium and potassium in cell physiology has evolved considerably. In an earlier era it was commonly thought that these ions move in a biological system in the direction of their concentration gradient, and under the influence of any electrical field in the neighborhood unless restrained by the mechanical opposition of some membrane with which they might interact in a passive fashion. This concept was based upon our inability to understand how alkali metals could interact chemically in any way except as completely dissociated ions in aqueous solution. We now have accumulated a considerable body of circumstantial evidence that potassium can be accumulated actively and sodium excluded from cells. This evidence has been reviewed elsewhere (Sheppard, '51) and a repetition here is not warranted.

In the case of red cells, data are found which suggest that the imposition of large concentration gradients will cause the slow passive movement of cations across the cell membrane. However, these movements are superimposed upon the normal cation kinetics and, so to speak, force the membrane to yield against its normal tendency to control the ions. That such cation control is necessary has been argued from time to time by postulating that the classical Donnan picture of ion distribution is a crude but useful first approximation to the true facts. We recall that the cell contains internal colloidal anions to which the membrane is impermeable. Under this approximation a redistribution of the permeable anions and cations results. The movement of the cations prevents the balancing of the inner and outer osmotic pressures and the cell swells to ultimate rupture. On the classical picture it was necessary to postulate that the cell was impermeable to cations and, therefore, stable.

We now have conclusive evidence that the membrane does pass potassium ions slowly. Figure 1 shows data obtained in our laboratory on the penetration of radioactive potassium into human cells (Sheppard and Martin, '50). We see that the isotope which was initially introduced into the plasma soon penetrates into the cells. From such experiments average rates of penetration can be calculated and, from this, rates of exchange of potassium between cells and plasma, since the plasma potassium can usually be held fairly constant in these experiments. The data in this figure yield a rate of 1.6% of the cellular potassium per hour at 38°C. Experiments at different temperatures give a smooth variation in rates with temperature seen in figure 2. Further studies show that the rate is nearly independent of plasma potassium concentration and depends roughly on the amount of intracellular potassium when different species are studied. In these experiments, others done by our colleagues at Harvard Medical School (Raker et al., '50), and pioneer studies at the University of Rochester by Dr. Fenn's group (Dean et al., '41), the reality

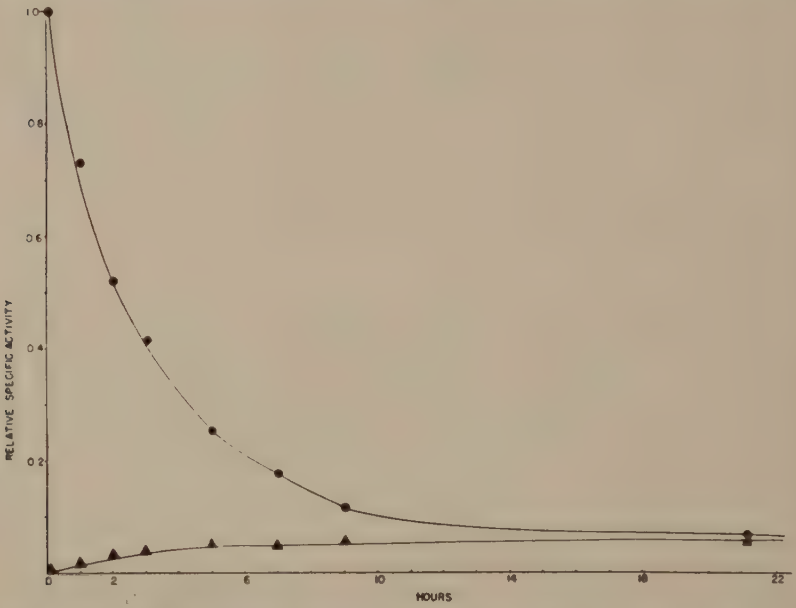

Fig. 1 Relative K^{42} specific activity versus time for cells (▲) and plasma (●) of human blood. Ordinates are specific activity (counts per minute per milliequivalent of potassium) divided by initial plasma specific activity. (Sheppard and Martin, '50.)

of cation exchange in mammalian red cells is established. We thus conclude that the red-cell membrane is slowly permeable to cations but that there are processes occurring therein which control the concentration of these ions in the cells. The osmotic catastrophe ensues only if these controls are disturbed by processes injurious to the cell.

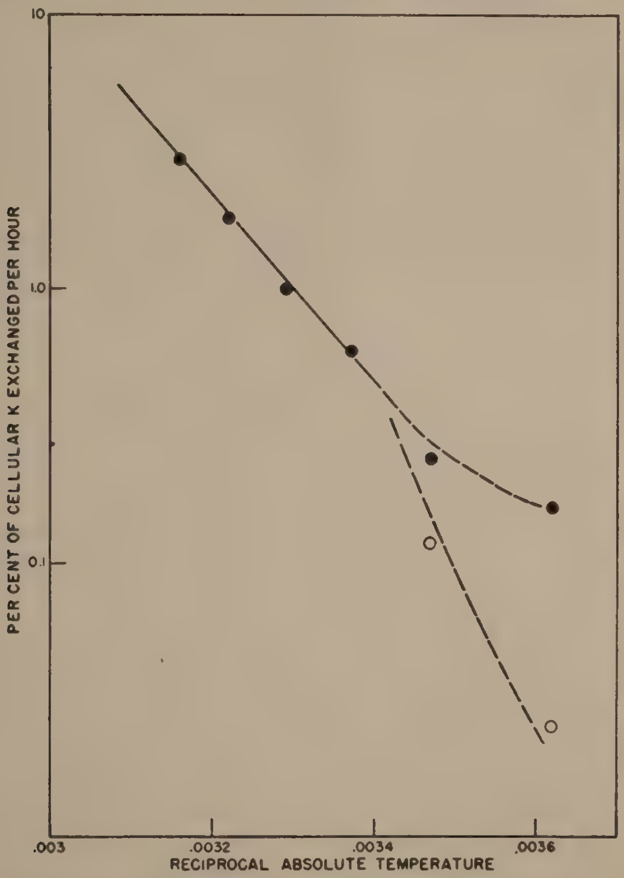

Fig. 2 Semilog plot of erythrocyte exchange rate versus reciprocal of the temperature. At low temperatures the rate from cells to plasma (●) exceeds the rate from plasma to cells (○). (Sheppard and Martin, '50.)

The nonpassivity of potassium in the red cell can be shown in experiments which relate the potassium accumulation to metabolic processes. It has been known for some time from the experiments of Wilbrandt ('37) and others that the metabolic poison, fluoride, will cause potassium to leak from red cells. We also know that when blood is kept at low temperature potassium is lost from the cells and regained upon returning the cells to physiological temperature, provided that sugar is present (Harris, '41). In this case the process requires the expenditure of energy. In most of these disturbances, if they are not carried too far, the loss of potassium is accompanied by an almost reciprocal penetration of sodium. It is of interest that similar disturbances have been recognized recently in a number of other animal tissues besides the red cell, suggesting that we have here a rather general phenomenon (Sheppard, '51).

EARLY RADIATION STUDIES WITH ERYTHROCYTES

The background literature on the study of radiation effects in erythrocytes is not extensive. It was known in the very early days of radium that blood could be hemolyzed by exposure to radium rays (Henri and Mayer, '04). Somewhat later, in 1911, the conversion of erythrocyte hemoglobin to methemoglobin with doses of several hundreds of thousands of equivalent roentgens of α particles was reported (Chambers and Russ, '11). This work also suggested that the effect was the result of the action of radiation on the cells and not the production of a hemolytic substance in irradiated plasma. Hemolysis and hemoglobin alteration by X rays was not described until Holthusen's work appeared several years later (Holthusen, '23). At this time it was noted that a time lag ensued between the radiation exposure and the lysis of the cells. A clue to this effect was obtained three years later when the work of Lehmann and Wels ('26) appeared which described the increased leakage of ions from irradiated bovine cells. It now became increasingly evident that the irradiated cells had suffered some sort of osmotic disturbance, and this was finally conclusively established in 1940 by Ting and Zirkle who showed that at 33,000 r exposures the X-ray effect was not due to the production of hemolytic substances but rather to a disturbance within the cells (Ting and Zirkle, '40a). This disturbance resulted in a slow swelling of the cells when kept at low temperature in serum following radiation exposure. Since it had been suggested that this swelling might be related in some way to the production of increased osmotic pressure within the cells due to the splitting of large molecules, experiments were performed to test this thesis, with essentially negative results (Ting and Zirkle, '40b). Studies of volume changes occurring when the cells were suspended and irradiated in large volumes of salt solutions showed that the irradiated cells had, to a considerable degree, lost their ability to prevent passive penetration of ions (Ting and Zirkle, '40c). Shortly after this, Wilbrandt and his associates in Switzerland reported observations by a somewhat less direct method which confirmed the effect and showed that it was considerably enhanced when the cells were stored at low temperature after irradiation (Liechti and Wilbrandt, '41).

Little was done further in this field until 1947 when Ting performed some experiments with radioactive potassium in irradiated cells. He was able to show that, under low temperature equilibration (5°C.), red cells exhibited electrolyte disturbances at as little as 400 r. Encouraged by Dr. Ting's findings and assisted by information which he generously shared with us, we began to work with erythrocytes in our laboratory.

RECENT ISOTOPE STUDIES WITH NONIRRADIATED BLOOD

To gain experience we first embarked upon a program of study with the isotope K^{42} in a nonirradiated control system. The important feature of the studies was the increased care taken to keep the cells in as nearly a physiological state as possible, since we felt that only in this way could we hope to improve on earlier work. We soon obtained a good deal of experience since we began with a more difficult task, working with dog cells, which we now know to have considerably less ionic stability *in vitro* than those of man.

Our methods have been described in more detail elsewhere (Sheppard and Martin, '50). One function of the lung was approximated by controlling the atmosphere above the blood. Similarly, in attempted imitation of one role played by the liver, dextrose was added to raise the sugar to 150–200 mg %. All the plasma constituents were retained and clotting was prevented with minimal amounts of heparin, sometimes a fairly pure crystalline solid heparin preparation being used.

TABLE 1

Typical record of hematocrits and plasma potassium concentrations of samples at the time of analysis

SAMPLE NUMBER	TIME AFTER TAGGING	HEMATOCRIT	CONCENTRATION OF POTASSIUM IN PLASMA
	min.	%	*mg %* [a]
0	2	40.3	17.8
1	17	40.3	18.2
2	35	40.3	18.2
3	49	39.9	18.2
4	66	40.5	18.5
5	90	39.9	19.2
6	124	41.1	20.0
7	188	41.0	19.5
8	304	41.0	22.5 [b]
9	420	40.7	21.6 [b]
10	540	41.6	20.9

[a] The values are uncorrected for viscosity error and sodium interference in the flame photometer.

[b] The sample had wet the wall of the flask during the experiment.

It is to be admitted that the only satisfactory proof of viability in the red cell would be a verification of its successful survival after transfusion, a test which is extremely laborious to make. Lacking this, we did the next best thing and followed, during the course of the experiment, the degree of hemolysis (usually very small) and the amount of swelling of the cells by determining packed cell volumes. Routine analyses of potassium were performed on cells and plasma of all samples. Table 1 shows a fairly representative set of results obtained on samples of dog blood. In human blood these indices usually showed good stability for considerably longer periods.

After some experience had been gained with unirradiated blood, radiation experiments were begun with human cells which are high in potassium and low in sodium (Sheppard and Beyl, '51). Surprisingly enough it was found that, if freshly drawn blood was irradiated at room temperature and then equilibrated at 24° or 38°C. under the carefully controlled conditions, radiation hemolysis was not observed. Table 2 shows a set of data obtained under the best conditions. At 43,000 r and after nearly 26 hours, the volume of packed cells had scarcely changed and significant hemolysis over that of the controls was not detected. The true situation became evident upon inspection of the analytical data (fig. 3, table 2). Potassium was leaking from the irradiated cells and sodium was penetrating almost reciprocally. It thus became obvious that, under the more physiological conditions, the initial disturbance of red cells in this dosage range is not radiation hemolysis at all. Since Ting and Zirkle found definite swelling at 33,000 r when blood was equilibrated at low temperature,

TABLE 2

Equivalence of potassium leakage and sodium penetration

	TIME	HEMATOCRIT	Na CONCENTRATION		K CONCENTRATION		TOTAL (Na) + (K)	
			Cells [a]	Plasma	Cells [a]	Plasma	Cells	Plasma
	hr.	*%*	*mM/liter*		*mM/liter*		*mM/liter of blood*	
Irradiated samples (43,000 r)	1.25	34.8	18.5	145	126	6.7	78	99
	2.50	35.7	21.8	138	122	8.1	79	94
	19	35.4	41.5	129	105	19.0	80	96
	25.6	35.1	46.0	127	102	20.2	81	95
	42	. . .	. . .	. . .	. . .	. . .	. .	. .
Controls	1.25	34.2	15.8	149	140	5.5		
	2.50	35.2	12.8	153	143	5.2		
	19	37.0	15.6	152	140	5.7		
	25.6	. . .	. . .	. . .	. . .	. .		
	42	36.0	19.1	150	142	5.1		

[a] Concentrations per liter of cell water assuming this to be 65% of the total cell volume.

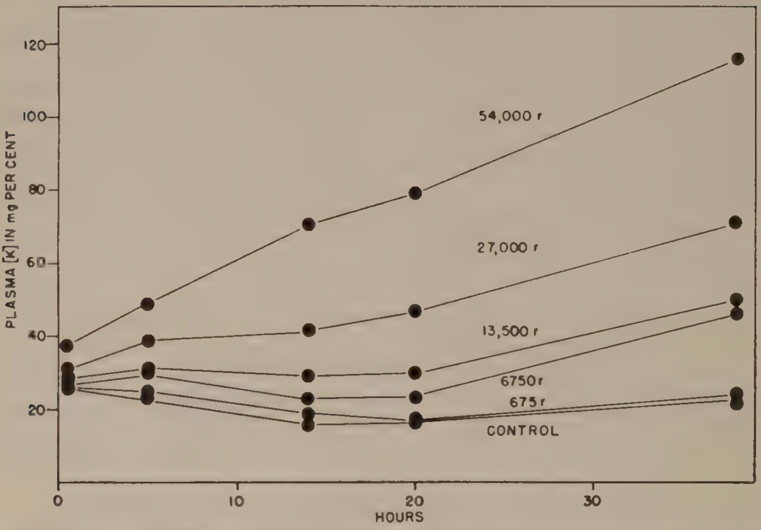

Fig. 3 Plasma potassium changes following exposure of human cells to various radiation doses. (Sheppard and Beyl, '51.)

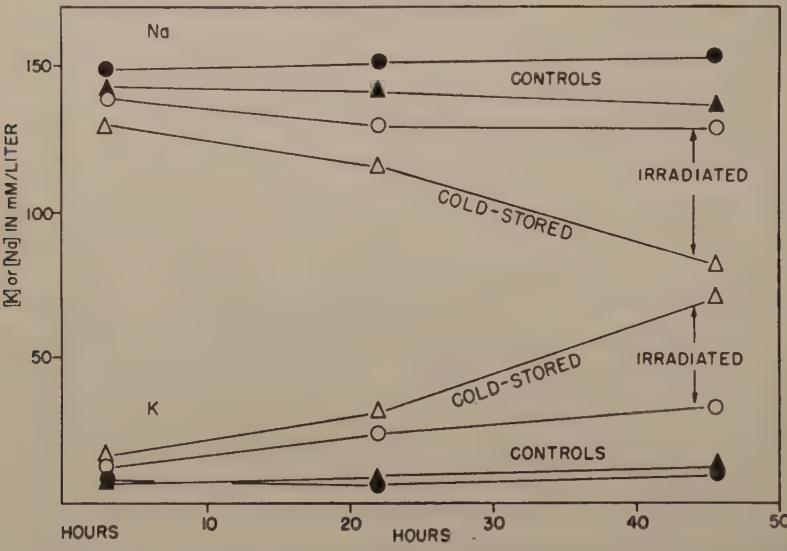

Fig. 4 Accentuation of radiation effect by low temperature equilibration (5°C.) following radiation exposure. (Sheppard and Beyl, '51.)

samples were equilibrated at 5°C. following irradiation at room temperature and it was found that the potassium leakage in human cells was considerably accentuated (fig. 4).

If there were not already a considerable body of information on reciprocal sodium-potassium exchanges in red cells produced by other disturbing influences, the importance of these observations might not have been realized. Potassium loss from red cells occurs as the result of exposure to glycolytic inhibitors (Green and Parpart, '50), silver (Davson and Danielli, '38), low concentrations of lysins (Ponder, '47a), and photodynamically activated dyes (Davson and Ponder, '40). It has been shown more recently, particularly in Ponder's work, that this potassium loss is accompanied by sodium penetration (Ponder, '47b), often almost reciprocally. Added to these observations are the recent ones by Overman who observed *in vivo* the exchange of sodium for potassium in the nonparasitized red cells of the malarious monkey (Overman and Davis, '48). We also recall that the leakage of potassium from red cells at 5°C. is accompanied by sodium penetration (Harris, '41). Parpart and Green ('52) discuss the phenomenon further in their review elsewhere in this symposium. Our observations thus add to this list the production of the effect by high doses of X rays. Our efforts to improve the viability of the system were rewarded in that the effect can be distinguished from a failure of total cation control, since the exchange process often precedes the osmotic catastrophe by a number of hours.

STUDIES OF RADIATION EFFECTS WITH ISOTOPES

Experiments were next undertaken to investigate the nature of the potassium leakage by tracer methods. Obviously potassium leaves the cells either as a result of accelerated outgo or, conversely, as a result of impaired uptake. By labeling the plasma potassium with the radioactive isotope, some information as to which process was occurring could be obtained. Figure 5 shows the result. It is seen that at 24°C. the rate of penetration, as shown by the rate of disappearance of the label from the plasma, is about 4.2% of the plasma potassium per hour and is unaltered by 35,000 r. In figure 6 is shown the over-all potassium leakage which was occurring meanwhile at about 12% per hour; thus, the outgoing rate was increased from 4.2 to 16% per hour. Such would be the case if some obstacle to potassium exit was removed which, when present, normally caused the potassium accumulation.

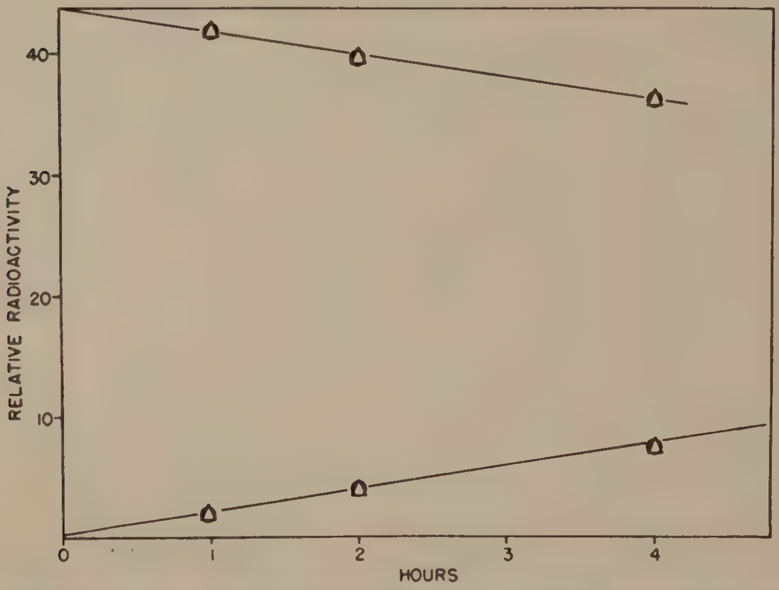

Fig. 5 Equality of penetration of radioactive potassium into irradiated cells (35,000 r, ●) and controls (△). (Sheppard and Beyl, '51.)

Sodium movement in human cells was next investigated. Here where the cellular sodium is low, if a small amount of sodium is tied more tightly than the rest, to intracellular materials, the binding will become evident because of the low amount of rapidly exchanging sodium with which the bound constituent must compete. Figure 7 shows a set of curves of the amount of radioactive sodium versus time in the plasma of control and irradiated samples of human blood at 38°C. We see that there is an initial rapid exchange but that a part of the sodium does not exchange appreciably, seemingly being stuck in the cells in some way. After radiation exposure the usual leakage of sodium into the cells occurs and with it goes some radioactive sodium, as the curves show. More detailed analysis shows that most of this radioactivity penetrates in this manner, little being involved in exchange. Furthermore little change is observed in the rate of the rapidly exchanging sodium. One particularly interesting fact was obtained — as radioactive ionic sodium progressively penetrates the cell the rapidly exchanging sodium is the portion which increases. This fact becomes apparent from the increase in the size of the hook in the figure, and strongly suggests that the rapidly exchanging cell sodium is ionic in character.

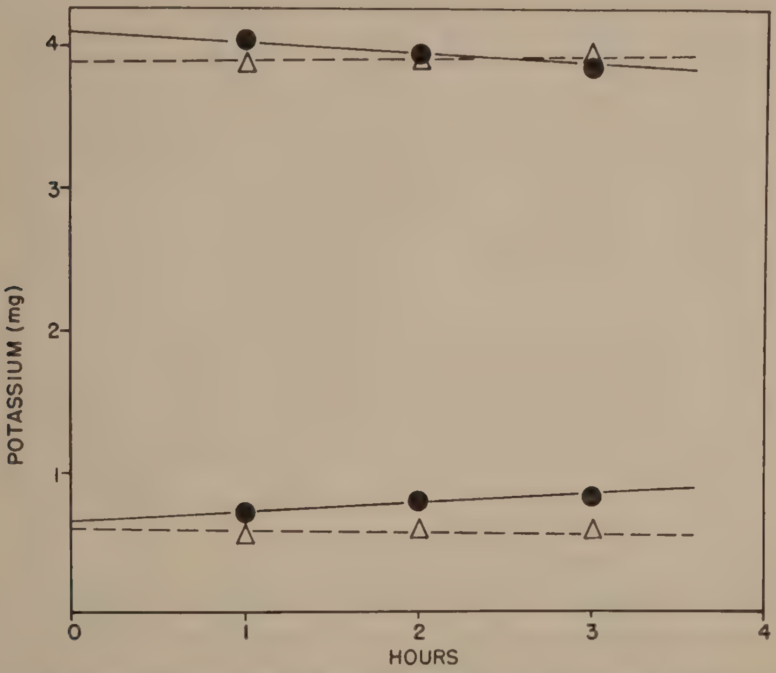

Fig. 6 Total potassium (mg) in cells (upper curve) and plasma (lower curve) of control (△) and irradiated (●) blood showing simultaneous loss of potassium at 23.9°C. from cells during uptake of K^{42} (fig. 5). (Sheppard and Beyl, '51.)

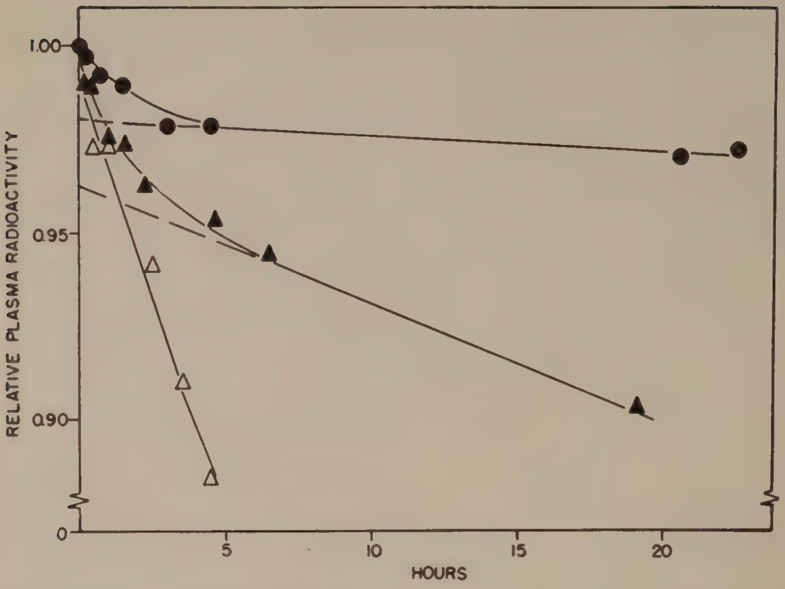

Fig. 7 Disappearance of Na^{24} from the plasma of control and irradiated blood. The ordinate scale is interrupted, showing only the greatly magnified upper portion. The control points (●) are those from a typical experiment on nonirradiated blood. The irradiated samples were exposed to 42,000 r, one group being kept at 38°C. for 6 hours (▲) and a second for 17½ hours (△) before introducing the isotope. (Sheppard and Beyl, '51.)

DOSE VERSUS EFFECT

The radiobiologist is generally interested in obtaining quantitative relations between radiation dosage and biological effect, and in trying to interpret them in terms of radiation action. In figure 8 we have plotted the excess of plasma potassium over the controls. It is seen that the leakage rate is not constant, and the curves for the higher doses tend to level off. This characteristic is also observed in the potassium-sodium exchange occurring in the presence of hemolysins (Ponder, '47b). A rough idea of radiation effect is obtained by passing second degree curves through the experimental points and determining the mean rate of potassium loss in each case. This is plotted in figure 9 against radiation dose, the vertical bars extending from the maximum to the minimum rate. The rate of loss is roughly proportional to dose, and at 20,000 r and 24°C., it is about 0.4% of the initial cell potassium per hour, approximately doubling for every 20,000-r increase. There seems to be little evidence of a threshold effect. It would seem to be premature at the present to attempt to account for the shape of these curves until more experimental information is available. One possibility which must be explored is that the radiation produces an elimination of sites of accumulation in the system. With fewer accumulation sites the total cell potassium would decrease to a lower equilibrium value.

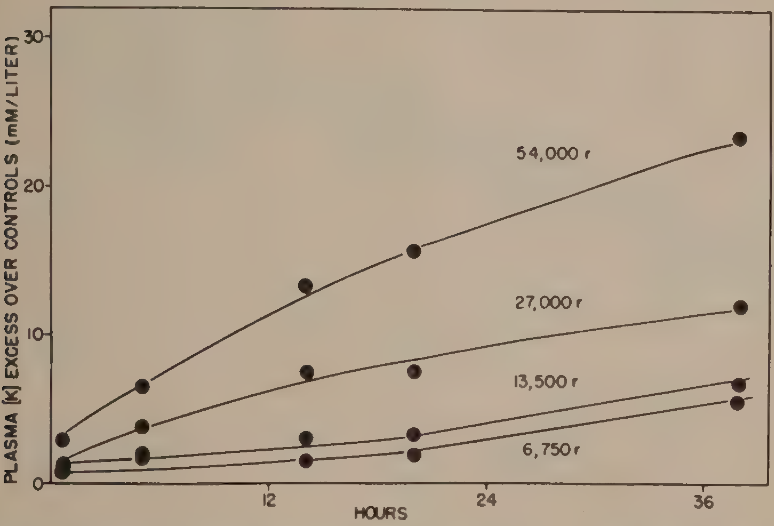

Fig. 8 Excess of plasma potassium over controls for the experiment shown in figure 3. (Sheppard and Beyl, '51.)

THE LACK OF AN OXYGEN EFFECT

In studies of radiation effects in several other systems, particularly those of Giles and Riley ('50) on the production of chromosome interchanges in *Tradescantia* microspores, irradiation under reduced oxygen tension lowered the radiation effect about threefold. To investigate the effect in erythrocytes, samples of whole blood were placed in 4-inch-long plastic tubes whose ends were closed with rubber diaphragm stoppers (fig. 10). Gas was passed over the blood through injection needles whose ends were kept above the fluid level. Samples were equilibrated with gentle rocking at room temperature, one set under 5% carbon dioxide and 95% oxygen, and the other under 95% helium instead of the oxygen. After equilibrium was reached, blood was delivered into a plastic absorption cell containing the same gaseous atmosphere. A thin layer was maintained in the absorption cell by a foil spacer, and by quickly freezing and thawing the erythrocytes to produce hemolysis, good optical transmission was obtained in a spectrophotometer. By taking the ratios of the optical densities at

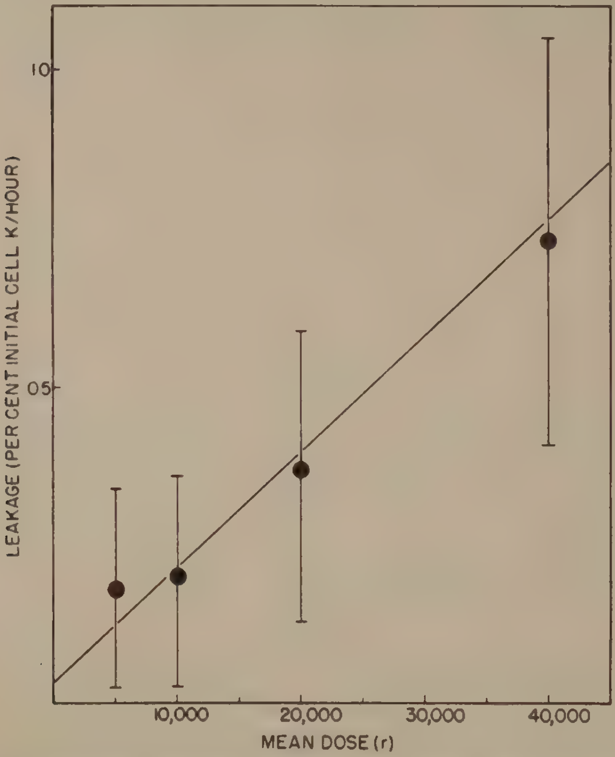

Fig. 9 Approximate potassium leakage rates versus dose for human erythrocytes. (Sheppard and Beyl, '51.)

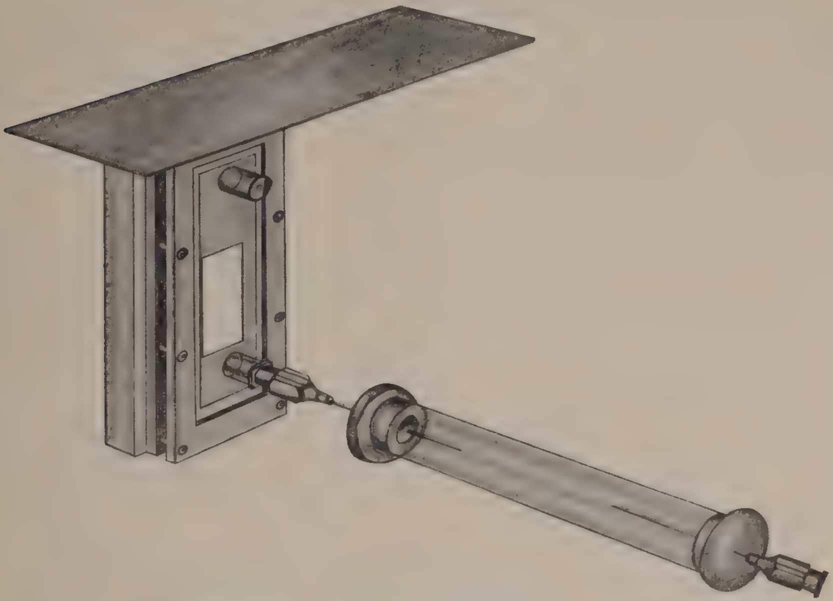

Fig. 10 Equilibration tube and spectrophotometer cell for studying effects of variable oxygen tension on radiation effects in red cells.

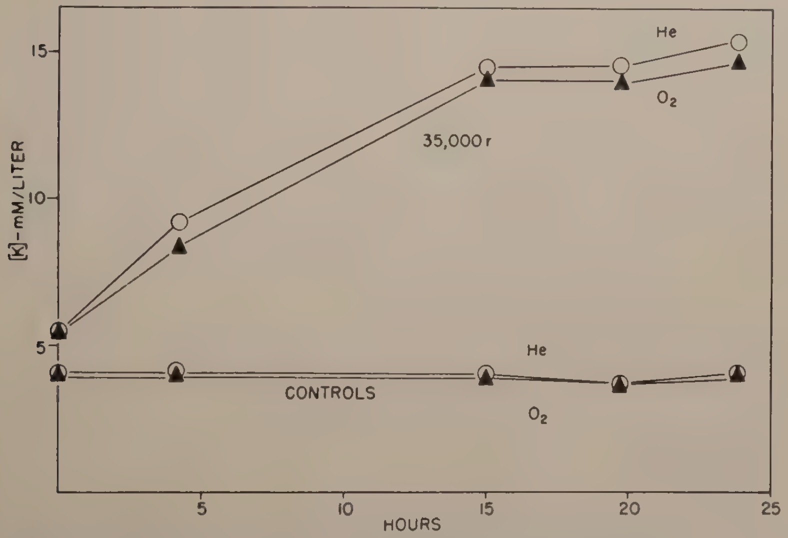

Fig. 11 Potassium loss from cells irradiated under 95% oxygen (▲) and 95% helium (○).

478 and 520 mμ it was possible to establish the quantitative removal of oxygen in the helium samples and the presence of oxygen in the control set. In figure 11, where the resulting potassium losses are compared, there is no evidence for a depression of radiation effect by the elimination of oxygen.

EFFECT OF RADIATION ON GLYCOLYSIS

Increasing evidence that selective potassium accumulation is related to biochemical processes, prompted a few preliminary observations of the effect of radiation on the metabolism of erythrocytes. Previous investigators have reported that high doses of X rays have little effect on the utilization of sugar by mammalian erythrocytes (Davis et al., '50). Figure 12 shows a set of determinations of sugar and lactate on samples of human blood equilibrated at 38°C., some nonirradiated and some exposed to 50,000 r of X rays. It is seen that there is no significant decrease in the rate of sugar utilization and lactate production. In one or two other experiments, we noted a possible slight increase in the rate of sugar consumption in the irradiated blood. This we hope to investigate further. An additional suggestion that the glycolytic cycle is substantially intact following large radiation exposures comes from determinations of adenosinetriphosphate (ATP) by the response of the isolated firefly luminescent system to this substance (performed with the assistance of Dr. Bernard L. Strehler) (Strehler and Totter, '52). Although other substances may conceivably be included in the determination, ATP is the only substance presently known to produce the luminescent reaction. The results shown in figure 13 indicate that there is still a liberal supply of this energy-rich material in the irradiated samples for over 5 hours after irradiation. The initial low value in the irradiated samples followed by a later overshoot has been observed in other experiments and will be further examined.

Although the biochemical results are still incomplete they suggest that, if the glycolytic system is at all significantly affected at 50,000 r, the effect is in a direction other than an

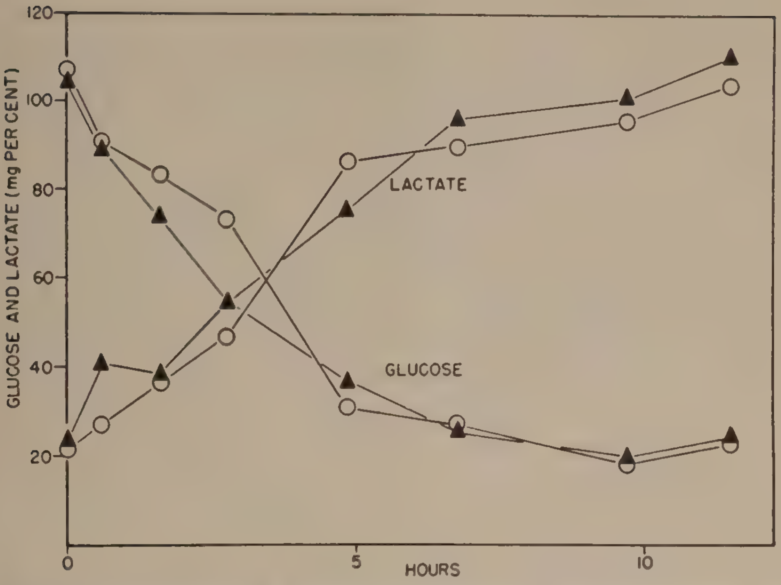

Fig. 12 Disappearance of glucose and production of lactate in control blood (○) and blood exposed to 50,000 r (▲).

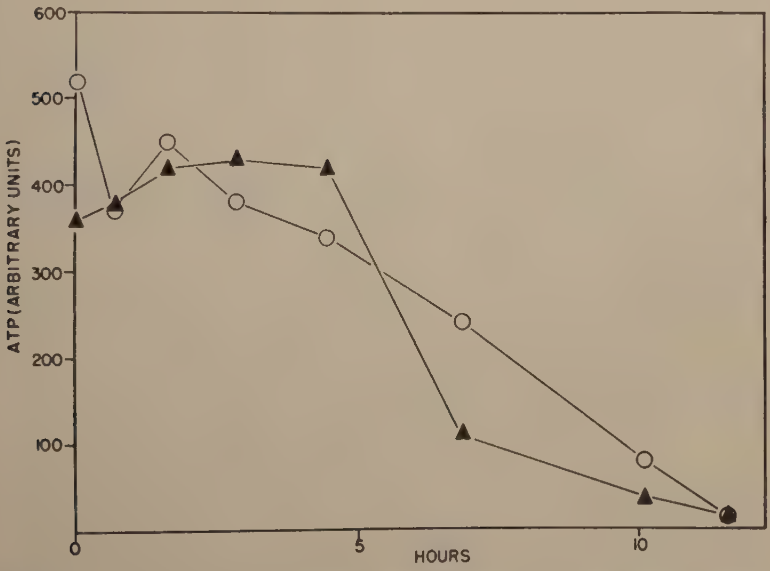

Fig. 13 ATP activity in irradiated cells (50,000 r, ▲) and control cells (○) *in vitro*.

inhibition. The several enzyme systems involved in the glycolytic metabolism still seem to be functioning with surprising effectiveness. We must, therefore, look for some other effect of radiation on the potassium mechanism than a simple depression of energy production in the cell.

EFFECT OF RADIATION ON CHOLINESTERASE

One of the most interesting recent biochemical observations has been that of Greig and Holland ('49) suggesting that potassium accumulation in erythrocytes is related in some way to acetylcholine turnover. With this in mind we have compared the activity of the cholinesterase in samples of control cells and cells exposed to 42,000 r of X rays (fig. 14). In this and other experiments, no significant difference could be found in the rate of hydrolysis of added acetylcholine after blood was exposed to radiation. That this hydrolysis is actually due mainly to the cholinesterase is shown by the inhibitory effect of added eserine. A residual hydrolysis is present but small.

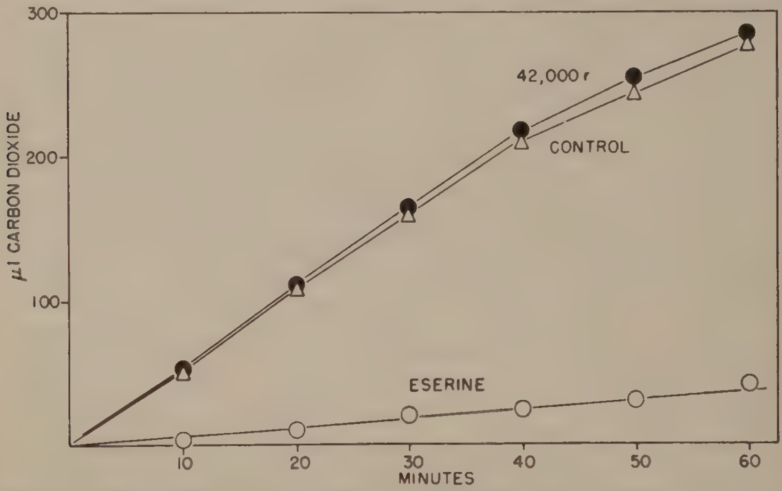

Fig. 14 Hydrolysis of acetylcholine by irradiated cells (42,000 r, ●) and controls (△). The blocking of the cholinesterase by eserine (○) is also shown. Ordinates represent CO_2 released from bicarbonate buffer by liberated acetate.

The negative result of the cholinesterase studies does not rule out a radiation effect on acetylcholine turnover. The isotope studies showed that the effect of radiation was to accelerate the outgo of potassium rather than to impair the uptake. In a recent report Taylor and Weller ('50) have stated that inhibiting the cholinesterase reduces the rate of penetration of potassium; whereas, acceleration of the outgoing rate is produced by substances known to inhibit choline acetylase, and this aspect of the problem awaits investigation.

ELECTROLYTE ACCUMULATION AND THE RED-CELL ENVELOPE

There is no experiment as yet which demonstrates unequivocally that ion selectivity occurs at the cell surface. Nevertheless this postulate gives the best explanation for a rather extensive body of experimental results. If the postulate is correct, radiation disturbance should produce changes in the cell envelope. Recently, electron microscope pictures of posthemolytic residues of irradiated and control cells showed a high frequency of gross alterations in the residues of the irradiated cells (Zacek and Rosenberg, '50). The original veil-like structure which might be the normal cell envelope is disrupted with the frequent production of holes. Although it is difficult to assess the ever-present hazard of artifacts in such observations the result is not inconsistent with the thesis of disturbance of the envelope.

It seems unlikely that the simple disturbance of selective potassium accumulation without osmotic hemolysis could result from direct perforation of the cell envelope. This would produce rapid loss of hemoglobin from the cell, and a characteristically different type of failure from that observed. It is more likely that the threshold injury occurs by a more subtle interference with a highly organized interfacial system which is potentially capable of active accumulation. The result might serve to accentuate the disruptive procedures involved in producing the posthemolytic residues.

If the disturbance of ion selectivity occurs at the cell surface, then an erythrocyte which is locally irradiated should fail locally at the site of exposure. This is difficult to demonstrate with small cells but recently Buchsbaum and Zirkle ('49) were able to irradiate local portions of the large nucleated red cells of *Amphiuma* with small micro sources of α particles and to demonstrate a local failure. One interesting phenomenon which they observed at their doses of over 100,000 equivalent roentgens was an initial shrinking of the cell prior to a later swelling and hemolysis.

CONCLUSION

It is well to consider where we now stand in this still relatively new branch of radiobiology. Firstly, a new type of cell disturbance by ionizing radiation can be definitely accepted. This disturbance seems to be only distantly related to problems of cytogenetics and to matters of processes in cell nuclei. The loss of potassium selectivity is a specific sort of cell failure and, in human blood, occurs well in advance of radiation hemolysis which can be delayed by maintaining the system in a quasi-physiological state. It is a failure of a basic property of the red cell and, in common with many radiobiological disturbances, it clearly has the character of a disorganization. The fact that specific potassium accumulation is a widespread property of living tissues means that the disturbance is of general biological interest. The preliminary results which we have obtained stress the importance of biochemical studies of the radiation disturbances of cells. Although of limited practical application at the moment, these studies represent a small contribution to the basic information being accumulated on the biological effects of radiation.

DISCUSSION

CHAIRMAN CURTIS: Dr. Sheppard's paper is open for discussion.

PARPART: Dr. Sheppard is to be congratulated on very interesting results. I would like to ask two questions, one concerning his ideas of a mechanism by which the retention in the cell in this double-phase affair might be possible; and second, if he wants to speculate at all — and I realize in talking with him that it would be a pure matter of speculation — on the surface properties that might account for his ideas or his results with respect to the passage of ions.

SHEPPARD: The Conway group recently, as you know, published a paper in Science in which they postulated a redox pump mechanism at cell interfaces. With my limited knowledge of biochemistry, I do not see how a redox pump would operate in the mature mammalian red cell since, as far as I know, there is no cytochrome in the red cell.

I do not see how potassium accumulation in the red cell can occur by simple exclusion of sodium, because if you say that sodium is excluded and therefore potassium goes in, you must stipulate that there is something which requires the sum of cations to be the same in the cell, which is merely another way of saying that the concentration gradient is maintained by active control of both cations.

I think we are beginning to look now for possible energy sources for this process. The process does require energy. If there is any leakage whatsoever of potassium from a red cell, then, if the cell is to maintain a constant level of potassium, it must get some of it back by an active process, which means accumulating potassium against a concentration gradient. As we know, this is an organizing process and requires a source of chemical free energy. We could speculate that this source of chemical free energy comes as indirectly from glycolysis in the cell, but that this has to go through the production of energy-rich phosphate bonds in some way. Then it may be that if the acetylcholine results are correct, the energy comes in through the fact that it takes free energy to put acetylcholine together, and when acetylcholine is hydrolyzed, this free energy becomes available again. Perhaps Dr. Nachmansohn will make some comments about this point later. If this acetylcholine mechanism is a real thing in the red cell, then perhaps the red cell is a good place to study these processes which are of such great importance in the physiology of excitable tissues.

BLINKS: Since the Conway proposal has been mentioned, I might point out a piece of negative evidence which was published 10 years ago. Suppose there might be systems within the cell surface, enzymes or otherwise, responsive to electron transfer, which is what he implies, one ought to be able to pick that up by bioelectric measurements. Of course Lund has been suggesting this for many years.

We tested this directly in the case of *Nitella,* which happens to be a good potassium accumulator and also tolerates anoxia. Over a range of 0.8 volts redox potential change (as shown by a platinum electrode), there was no more than a millivolt or two shift in the bioelectric potential; which however was meanwhile quite responsive to potassium concentrations.

SHEPPARD: Do you think there is an active accumulation mechanism for potassium in plants?

BLINKS: I think there must be. It is accumulated against an immense gradient in the case of *Nitella,* not only of potassium versus sodium, but of total salts over the environment. The cell contains some 0.1 *M* salt, largely potassium chloride; whereas, the environment, muddy ponds or otherwise, contains perhaps 0.001 *M* or 0.0001 *M* of salts. Such accumulation must be active.

PARPART: There is a difference here, however. Just as in the case of plant cells, animal cells show an accumulation. One man will analyze liver cells or red cells which will show a very definite accumulation in an environment that is high in sodium.

However this accumulation, in the case of animal cells as well as in plant cells, is a matter of formation during development. Accumulation in the mature cell, if you can imagine that status ever having been reached, is a fixed thing rather than what occurs during the development of the cell, which is very typical of plant cells. It may be similarly typical of animal cells.

SHEPPARD: If the isotope can get through the red cell membrane, then, presumably, it can leak out under the concentration gradient over many weeks; and the only alternative is that there is some mechanism whereby every time one potassium atom leaks out under the concentration gradient, free energy is conserved and re-utilized to get another potassium atom back.

PARPART: There is another hypothesis that needs testing, namely, that the radioactive potassium might well be in the membrane. I do not know of any evidence that the potassium is distributed throughout the entire cell, rather than in the ghost. It seems to me, it would be very profitable to get a picture of what the ghost is doing. Once that is settled, then I think the next point would be the matter of whether it is an active accumulation of radioactive potassium.

SHEPPARD: There is plenty of room for all the potassium in the red cell membrane, if you take the classical ionic radius as any indication of the ionic spacing. If you postulate that the exchangeable potassium is all on the membrane then you will postulate that all the potassium is at the interface, because the radioactive potassium exchanges to completion. Then the only cation which is in the cell is a small amount of sodium, and one must strive for some mechanism to maintain the osmotic pressure of the inside of the cell, or postulate that there must be some resistance to water transfer of some other mechanism for maintaining the osmotic pressure in the red cell, since it cannot support any great amount of mechanical stresses in the membrane.

GREIG: If we suspend human cells in a bicarbonate medium they lose potassium. The same thing happens with dog cells. After they have lost a certain amount of their potassium, if we add acetylcholine, we get a replacement of potassium; whereas, potassium in the control system continues to go down. The accumulation occurs against a concentration gradient.

This happens if we add acetylcholine, and other esters which are metabolized by the cholinesterase of erythrocytes. If we add benzoylcholine which is not metabolized, the accumulation does not occur. So this picking up of potassium, whether it is putting it back in the membrane or whatever the cell does with it, seems to be related to the activity of the cholinesterase enzyme.

WILDE: In tissue work we always consider reversibility. When external K concentration is lowered, the sodium goes in, and often in muscle, we can show that it will come out again when the external K is raised.

This suggests that there might be an active process involved; and we do not have the feeling that in muscle the thing grows up that way with accumulated K and excluded Na, and closes the door, but that the ion pattern is something that is being continually maintained by active processes.

In the case of the corpuscle, I wonder if there was ever any suggestion that sodium could be reversed? I suppose often, *in vitro,* you run into time and deterioration problems.

PARPART: I have never seen any reports on the reversal of sodium, and we certainly have never obtained any.

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THE EFFECTS OF RADIATION ON THE CYTOLOGY OF THE EYE[1]

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FOUR FIGURES

These studies, carried out in the Knapp Memorial Laboratory during the past two years, were undertaken for the purpose of investigating some of the phases of the pathogenesis of X-ray cataract, and the descriptions of cytologic changes in this report must therefore be limited to the changes occurring in the tissue elements of the crystalline lens. In its anatomy, biochemistry, and metabolism the lens differs greatly from other structures of the eye and from other organs, so much so, in fact, that the sequence of observed changes may bear little relation to the course of biological effects of radiation on other, dissimilar tissues. However, the avascularity of the lens, and the fact that it possesses a single layer of epithelial cells separated into two functionally different types, one of which produces the main bulk of fiber-shaped cytoplasm, provide conditions distinctly favorable to an analysis of radiation effects.

HISTOLOGICAL CONSIDERATIONS

It would seem advisable to preface this report with sufficient anatomical data to illuminate the peculiarities of the

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[1] The studies on the effect of roentgen rays on the eye, of which this paper is a part, are based on work performed under Contract AT-30-1-Gen-70 for the Atomic Energy Commission.

This study was also supported by the Knapp Memorial Foundation.

growth of the lens for which the equatorial zone is solely responsible. Lesions in this zone, involving both the epithelial cells and the nucleated fibers, precede, in all probability, the processes of proteolysis which characterize the formation of most nontraumatic cataracts. For this reason I take the liberty of illustrating at this point certain histological and cytological details of the lens equator which were worked out for us more than 50 years ago by Becker (1883), Henle (1878), and Rabl (1899). Since that time there have been, unfortunately, no new contributions to our knowledge of the histogenetic events in this locality, possibly because of the difficulties encountered in handling lens tissue by any of the established techniques. Rabl gave the most accurate description of the rabbit's lens, and his outstanding work, which has been considered fundamental since its publication at the end of the last century, is the basis for many of the following observations. No marked anatomical differences have been noted among the various species of mammals examined.

It should be recalled that the lens is enveloped by an elastic capsule and that the monolayered epithelium situated underneath the capsule ends rather abruptly at the equator, and thus covers only the anterior aspect of the lens. This sharp delimitation, marked by an intense red line, is readily demonstrable with the Feulgen technique for desoxyribonucleic acid (DNA) on the undissected lens. Except for a small peripheral zone, which in rabbits measures from 2 to 3 mm in width, the main portion of this epithelium seems to possess the characteristics of a resting epithelium. It is in the ring zone, near the equator, that growth of the lens takes place and continues throughout life by the transformation of the epithelial cells into nucleated lens fibers. It may well be that the greater part of the epithelium of the anterior surface nourishes the lens by a mechanism of active transfer of material from the surrounding aqueous humor.

Toward the peripheral or germinative zone, the flat cells of the central portion assume a cubical or cylindrical shape and increase in height to three times their dimension at the anterior pole (fig. 1). The irregular, cobblestone pattern of the middle section of the anterior surface gives way, behind the equator, to a monodirectional arrangement of cells in the form of short, meridional rows of about 8 cells. Between these meridional rows of the equator and the flat cells of the anterior aspect lies the equatorial zone of densely packed cells with round nuclei in which mitotic figures can be seen with regularity. The transition from the equatorial part of the epithelium to the cells which develop into lens fibers can be better illustrated than described. Figure 2 shows that

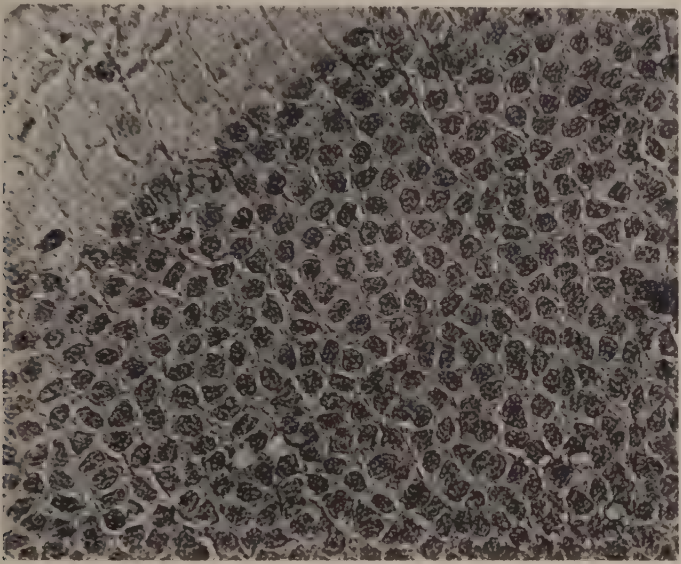

Fig. 1 Equatorial zone and meridional rows in lens epithelium of a normal rabbit (Feulgen).

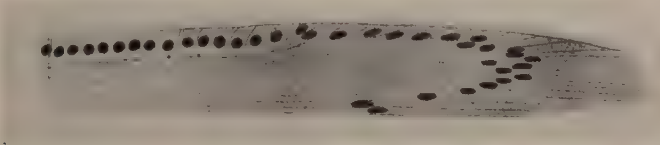

Fig. 2 Equatorial area of lens epithelium with bow (squirrel). Reproduced from Rabl (1899).

portions of the cells adjacent to the covering capsule become broader and form short pyramids. The more posteriorly situated cells insert at the capsule in an increasingly oblique fashion, which can be observed first in the outer half and then also in the inner half. In the cell elements which lie at the extreme periphery, this inner part appears elongated and stretched. The stretching process continues until the anterior end of the cell, i.e., of the young lens fiber, extends forward between the epithelium and the older fibers, while the posterior end is pushed more and more backward by the advancing cells of the equatorial epithelium. By torsion of the cells for 90° around their long axes, the nuclei now appear to have moved inward and forward in varying degrees and present, in meridional sections, the characteristic picture of the lens bow or vortex. The nuclei here are larger, oval, and more leptochromatic when compared with the nuclei in the pre-equatorial area. For our present purpose a more detailed description of these histogenetic processes would seem to be unnecessary.

MATERIALS AND METHODS

The various approaches which were selected for this study had the following elements in common: (1) the radiation factors used in a single exposure to 2000 r (table 1), (2) the experimental animals (mature chinchilla rabbits), and (3) the concentration of attention on the early stages of radiation damage to the lens. The animals were subjected to sodium pentobarbital anesthesia and fastened to the board so that one eye of each animal could be irradiated while the other, shielded, remained outside the beam. This second, control eye received from 5 to 7 r of stray radiation. Repeated observations by means of focal illumination with the biomicroscope and with the ophthalmoscope, have been made at intervals ranging from 24 hours to 2 weeks over observation periods varying in length up to 16 months. More than 100 animals have been utilized.

The two principal technical approaches by which the equatorial and pre-equatorial region of the lens can be studied under the microscope are (1) by means of thin meridional sections, and (2) by means of flat preparations of the epithelium with the capsule. Sections accurately placed in the meridional plane will reveal changes in the lens fibers as well as lesions in the nuclei of the pre-equatorial area, of the meridional row, and in the lens bow which belong to these fibers. In such sections, moreover, cell counts in corresponding areas of the equator in identically treated experimental and control lenses can be compared and the cytochemical changes evaluated accordingly. Sectors of the epithelium of well-fixed lenses can be procured by peeling and will provide satisfactory flat preparations (Rabl, 1899). Under favorable circumstances Verhoeff ('16) and recently Poppe ('42) obtained total flat mounts but it was impossible for Poppe to avoid drying effects and these misled him in the identification of a new type of cell. A few weeks ago Dr. Alma Howard (personal communications) made available to us a procedure which prevents these artifacts completely and appears to be extremely well suited for high-power microscopy of the surface cells of the elements in the pre-equatorial zone and of the meridional rows. Some stretching and distortion of the cells in these rows cannot be avoided, however, and the nuclei of the lens vortex and lens fibers cannot as a rule be visualized. Since the two methods supplement each other in a satisfactory manner, it is desirable to use them both.

TABLE 1

Radiation factors

220 kv	Filter: ½ mm Cu	TSD: 30 cm
20 ma	1 mm Al	HVL: 1.0 mm Cu
Field: 20 mm round		r/min. in air — 145

THEORETICAL CONSIDERATIONS

Before reporting on our results, I should like to remind you that there are, in the main, two opposing theories on the pathogenesis of X-ray cataract. According to the theory to which most authors subscribe (Poppe, '42; Bossuet, '09; Politzer, '29; Rohrschneider, '29, '30a, b, '32a, b; Peter, '30; Grzedzielski, '35; Okusawa, '37; Leinfelder and Kerr, '36; Goldmann and Liechti, '38; von Szily, '38; Krückmann, '40), the primary lesion is located in the cell nuclei of the germinative zone; destruction of the lens fibers, which finally causes the opacity, is considered a late change of secondary character. Proponents of the second hypothesis (Aulamo, '31; de Schweinitz, '32; Milner, '33, '34; Wölfflin, '38) suspect that changes in the permeability of certain phase boundaries, produced by the ionizing radiations, constitute the mechanism responsible for the development of the lenticular opacities, and in their opinion such mechanism would best explain the clinical picture of radiation cataract in man. As to the first theory, some agreement has been reached among experimental workers as to the onset of the first clinically visible signs in the periphery of the lens although contradictory observations have recently been reported (Goldmann and Liechti, '38).

EXPERIMENTAL RESULTS

Morphological radiation effects on the lens of the rabbit. The present extensive study provides, with some minor modifications, confirmatory evidence of the peripheral site of the early changes. When the pupil of the rabbit was maximally dilated with atropine and neosynephrine, the equator of the lens was found to be accessible for inspection with the ophthalmoscope. At this most peripheral zone a dark ring of granular structure appeared about 4 weeks after irradiation. Vacuoles later became visible at some distance from the equator, and in the following week, formed clusters which developed into a second ring opacity often characterized by a garland pattern. From the second ring, opacity extensions spread toward the posterior pole. The equatorial changes became less well defined so that in later stages it was difficult to identify them.

In former studies, lenses of eyes exposed to X radiation have rarely been examined before the appearance of lens opacities. Only in Poppe's work was sufficient attention placed on early changes in the lens epithelium. In our series the first definite deviation from the norm was seen in meridional sections in the form of a displacement backward, and sometimes forward, of some of the nuclei of the bow as early as one week (fig. 3). Clumping of chromatin, karyorrhexis, and atypical mitosis in the pre-equatorial zone seemed to occur at about the same time. It should be noted here that our knowledge of the mitotic activity of lens epithelium is meager and that the subject needs further study. A greater number of mitoses were observed in the equatorial zone than in the surface epithelium and, contrary to Rabl's experience, mitosis could be seen occasionally in the nuclei of the meridional rows. Our findings were sharply at variance with those of Poppe who reported that, in total flat preparations, only 5 or 6 dividing cells could be found. It may be said safely that in our preparations this number was multiplied by 20 or more.

Two weeks after irradiation the cells in the pre-equatorial zone were spaced less densely and less regularly and often showed swelling of the cytoplasm. Striking was the appearance of giant nuclei and multinucleated cells as they have been described by Poppe ('42) and Cogan ('51). After another week or two their palisade arrangement, as seen in the meridional sections, disappeared; they became flatter and the rows of nuclei showed broad interruptions. Cell counts of the germinative zone in paraffin sections illustrated the regression in the number of nuclei within the first two months after irradiation (table 2).

The first difference in the appearance of the lens fibers between irradiated and control eyes was noted two weeks after irradiation in the form of a slight increase of thickness

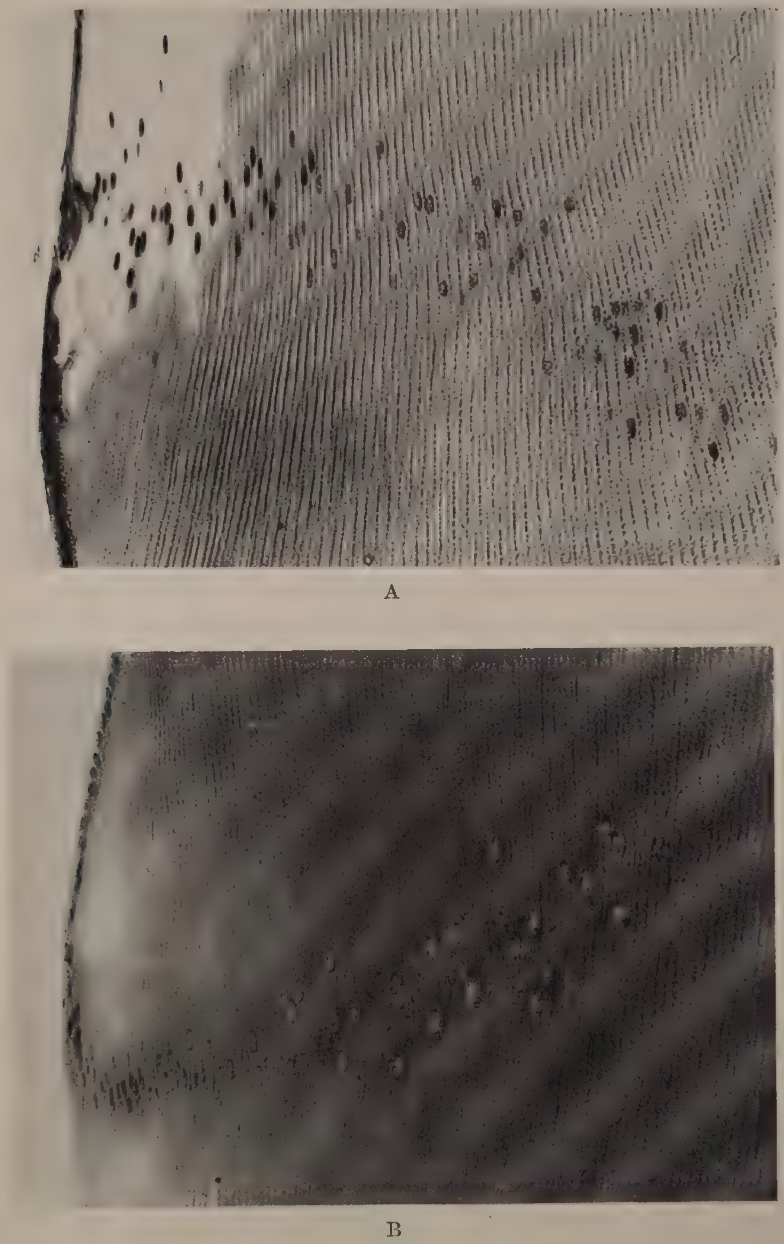

Fig. 3 Lens bow of control eye (A) and of irradiated lens (B) showing displacement of nuclei (Feulgen).

at the subcapsular ends. This was followed by a change in the angle of the insertion of the fibers on the capsule, which became less oblique both pre- and postequatorially.

It is of special interest that in 6 of a series of 56 eyes, large cystic spaces filled with granular matter were seen at the ends of the fibers of medium length 4 weeks after irradiation (fig. 4). By transillumination the same lenses exhibited *in vivo* brilliantly reflecting vacuoles along the posterior horizontal suture. As far as the histologic picture was concerned, the severity of the radiation damage at the subcapsular ends of the fibers contrasted with the moderate degree of the changes in the nuclei of the pre-equatorial zone and of the lens bow.

TABLE 2

Cell counts in pre-equatorial epithelium of irradiated and control lenses

TIME AFTER IRRADIATION (WEEKS)	NUMBER OF RABBIT LENSES	COUNTS [a]	
		Irradiated eyes	Control eyes
1	4	1. 72.66	1. 99
		2. 60.16	2. 76
2	4	1. 69.3	1. 91
		2. 57.33	2. 92
3	1	1. 64.2	(no control. 89.7 av. of all controls)
4	6	1. 47.3	1. 91
		2. 41.0	2. 86.66
		3. 56.0	3. 81.66
8	4	1. 63.0	1. 85.0
		2. 38.12	2. 103.66

[a] Each figure is the average of counts in three sections of each lens.

One could say, then, that the injurious effect of this type of irradiation on the rabbit's lens became visible microscopically as soon as one week after irradiation. It consisted of (1) the displacement of nuclei in the bow and (2) of structural changes of the epithelial cells in the germinative zone. These signs were thus in evidence 3 to 4 weeks before the ophthalmoscopic examination of the equatorial area revealed the first opacity in the form of a dark ring. Excessive formation of large cystic spaces at the ends of the lens fibers in 6 eyes, 4 weeks after irradiation, indicated at least an occasional primary damage of the cytoplasm under the conditions of the experiment.

Radiation effect on the formation of DNA. The reports of Ahlström, von Euler, Hevesy ('44, '45) and Stowell ('45) on the blocking of the formation of DNA in tissue by X rays stimulated experiments in which irradiated and control lenses were submitted to examination by means of cytochemical methods. The Feulgen reaction (Stowell, '45) and the methyl green technique (Pollister and Leuchtenberger, '49a, b) were employed on paraffin sections and on flat preparations of the lens epithelium in order to study the formation of highly polymerized DNA. In these preparations the nuclei of the germinative zone stained more deeply than

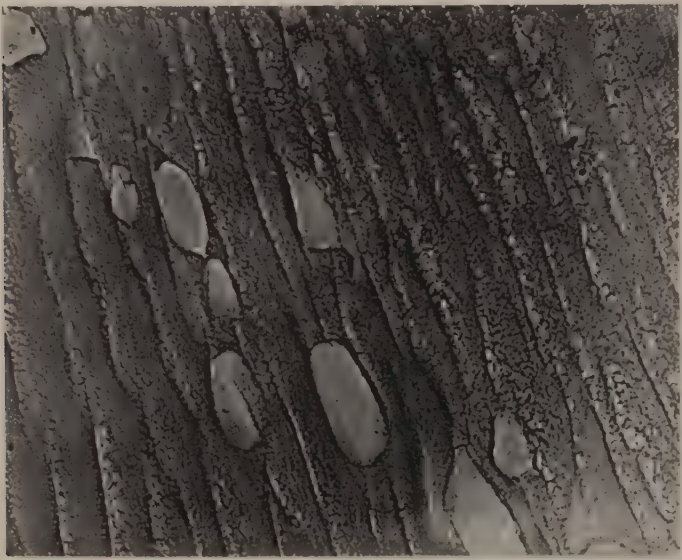

Fig. 4 Formation of cysts and vacuoles, after irradiation, in and between lens fibers in front of lens equator (H and E).

the nuclei of the anterior surface. Rabl explained this difference in staining intensity between nuclei of the central and peripheral areas as a reflection of the varying thickness of these nuclei in the respective parts of the epithelium. Experiments with the methyl green pyronin technique indicated, however, that physical factors alone could not account for this phenomenon. The slide shows that the content of DNA, especially in its highly polymerized form, is greater in the nuclei of the germinative area.

No difference in the intensity of staining between irradiated and control lenses could be discovered until two or three weeks after irradiation. By this time, however, structural changes had also developed. These observations are at variance with those of the authors last mentioned who described a decrease of newly formed DNA a few hours after exposure to penetrating X rays. In cautious terms it may be stated that in our preparations the alleged decrease in the turnover of DNA after irradiation could not be demonstrated at early intervals, and that whatever inhibition of newly formed DNA may possibly have existed did not alter the cytochemical reaction in the nuclei sufficiently to be quantitated by Gomori's ('50) technique on Coujard slides.

Radiation effect on the permeability of the lens. The last part of our study on early lens changes after X irradiation has included a rather extensive series of experiments dealing with disturbances in the permeability of the lens (von Sallmann and Locke, '51). For the purposes of this report it may suffice to review briefly the data from those experiments which have a bearing on the general aspects of the pathogenesis of radiation damage to the lens. Radioactive indicators (Na^{24}, K^{42}, I^{131}, and P^{32}) served as tools. Specific activities in the serum, in the aqueous humor, and in normal and irradiated lenses were measured by the standard technique or by densitometry on autographs, with the following results:

1. The uptake of radiosodium by lenses with X-ray cataract, but not by clear irradiated lenses, was greater than the up-

take by control lenses. It increased with the progress of the cataractous process, probably in connection with the development of an extracellular phase in the damaged lens.

2. Radiopotassium, like radiophosphorus, penetrated well into the normal lens. Four hours after intraperitoneal introduction of the labeled element (K^{42}) the ratio of the photographic effect of the anterior cortical layers and of the aqueous humor was greater than one. The exchange of radioactive potassium with the potassium of the cataractous lens appeared slightly decreased, but contamination of the bombarded potassium borate with sodium prevented the determination of the extent of the decrease.

3. The accumulation of radioiodine in irradiated lenses with initial cataractous changes, but not in clear irradiated lenses, exceeded the accumulation in normal lenses and suggested an augmented permeability of the damaged lens for this indicator.

4. Radiophosphorus accumulated in normal lenses principally at the equator and in the surface layers of the cortex. The differences between the cortical and deeper portions vanished in lenses with total radiation cataract but the autographs of normal lenses and lenses with initial radiation damage were alike.

SUMMARY

Beginning displacement of nuclei in the lens bow, clumping of chromatin, and nuclear fragmentation in the epithelium was observed about one week after irradiation with 2000 r. Both displacement and changes of the nuclei progressed in the following week. Thus the early irradiation effects seen histologically precede the development of clinically visible changes for several weeks. Among the early ophthalmoscopically visible signs are occasionally numerous vacuoles at the end of the lens fibers, which are of a transient nature and most likely the expression of a primary lesion of the cytoplasm.

In the experiments reported here it was not possible to demonstrate a decrease of the turnover rate of DNA within the first two weeks after irradiation.

In the experiments with radiosodium and radioiodine, the differences in radioactivity between control and irradiated lenses did not become measurable until initial cataractous changes had developed. These investigations did not, therefore, lend support to the theory that enhanced permeability of the lens or depressed processes of phosphorylation lead to the development of lenticular opacities produced by X rays. They suggested rather that these changes accompany and influence the progress of the opacification.

DISCUSSION

CHAIRMAN WARREN: This paper by Dr. von Sallmann is open for discussion.

KREBS: Your technique of irradiating one eye and shielding the other reminds me very much of the technique used by von Hevesy to measure the indirect effects of radiation. He grafted tumors on both sides of animals, irradiated the tumor on the left side, shielded the tumor on the right, and found also a strong inhibition of nucleic acid new formation on the right side. Nucleic acid metabolism and mitotic activity are believed to be closely connected; but up to now nobody has shown an indirect effect on mitotic activity.

In the course of our own experiments intestine of rats was surgically removed and shielded during irradiation, but no indirect effect on mitotic activity was found.

I would like to ask if you noted any evidence for an indirect effect on the mitotic figures or the mitotic activity in the shielded eye.

VON SALLMANN: Our knowledge of the mitotic activity of the lens is extremely limited at the present time. As yet we have not obtained data on the mitotic rate in various parts of the epithelium.

KREBS: Maybe there are also explanations why you could not find some effects on the nucleic acid metabolism under the chosen conditions. If von Hevesy is right, that mitotic activity and nucleic acid metabolism are so closely connected, then mitotic activity should be affected.

VON SALLMANN: First we tried to find effects of X irradiation on the turnover rate of DNA by comparing irradiated eyes of rabbits with the eyes of normal, nonirradiated animals. There were biological variations among animals in such a range that it was thought to be safer to use, as a control, the second nondirectly irradiated eye of the same animal, although it had received ~6–7 r of stray radiation. Stowell and Ahlström found small differences in mouse tumors when they shielded one flank. It is quite possible that such differences were too small to be recognized with the technique of quantitative estimations which we used in our experiments.

SPARROW: Doctor von Sallmann, what was your method of measuring the methyl green and Feulgen staining intensity? Was this the photometric method?

VON SALLMANN: No, it was a modified Gomori's technique on Coujard slides. It was felt that this method would be sufficiently accurate for our purpose.

SPARROW: I would like to mention that Dr. Moses in our laboratory has been doing similar work, using methyl green and Feulgen reaction up to 20,000 r, and has found no significant change in either plant or animal material, which does not agree with some of the other work already published.

FURTH: There are two types of exposures of major importance with respect to the cataract problem; notably, repeated small exposures over long periods of time, and single, massive exposure as encountered at atomic bomb explosions. May I ask you whether recovery is possible. The notion is prevalent that, if the damage is done following single exposure and reaches a certain stage, recovery will not take place.

I should very much like to know your experience concerning the progression and recovery following simple exposures of varying intensities.

von Sallmann: Our experience with divided or with small doses is rather limited. There is no doubt that the irradiation damage can be both decreased in intensity and delayed in appearance by either dividing the dose, say 2000 r, into two irradiations of 1000 r with an interval of three weeks or longer, or by lowering the dose. Animals which were irradiated with 750 r developed the first clinical signs of radiation damage 3 to 4 months after the irradiation instead of the 4 weeks which elapsed between the exposure to 2000 r and the first appearance of lenticular opacities.

The problem of recovery of tissue elements in a structure like the lens is extremely difficult to evaluate. Certainly we can say that some clinically visible changes, as for instance the accumulation of vacuoles near the horizontal suture and vacuoles at other places, disappear without residue. Thus, there must be some recovery taking place. On the other hand, it is felt in general that slightly damaged nuclei produce abnormal fibers which differ in their physical and biochemical make-up from normal fibers and become opaque after a latency period of years. I do not think I can answer the question of how far recovery plays a role in this process.

Warren: The early stages of the formation of the vacuoles, as you have shown them in the equatorial region, were within the substance of the fibers. As the vacuoles extended on into the lens substance and posteriorly, did they form extracellularly as a result of rupture and confluence of these vacuoles? Or is there any clue as to how the more dense vacuoles are formed?

von Sallmann: This is an important question. It was my impression that the smallest vacuoles were located within the fibers, intrafibrillary; whereas, the larger cystic spaces seemed to lie between the fibers. I thought that the vacuolar changes occurred first within the cytoplasm.

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VOL. 39 JULY 1952 SUPPLEMENT 2

CONTENTS

Introduction — ALEXANDER HOLLAENDER 9

L. R. BLINKS. Effects of radiation in marine algae 11

E. J. BOELL. The effects of radiations on respiratory metabolism. Three figures 19

ALEXANDER L. DOUNCE. The interpretation of chemical analyses and enzyme determinations on isolated cell components 43

DAVID E. GREEN. Organized enzyme systems 75

MORTON MCCUTCHEON. Problems and effects of radiation on capillary permeability. With discussion by Jacob Furth — Endothelial fragility by ionizing radiation. Three figures 113

DAVID NACHMANSOHN. Nerve function and irradiation effects. Eleven figures 137

A. K. PARPART AND J. W. GREEN. Some problems in connection with ion permeability of erythrocytes 179

C. W. SHEPPARD AND MARIAN STEWART. The direct effects of radiation on erythrocytes. Fourteen figures 189

LUDWIG VON SALLMANN. The effects of radiation on the cytology of the eye. Four figures 217

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